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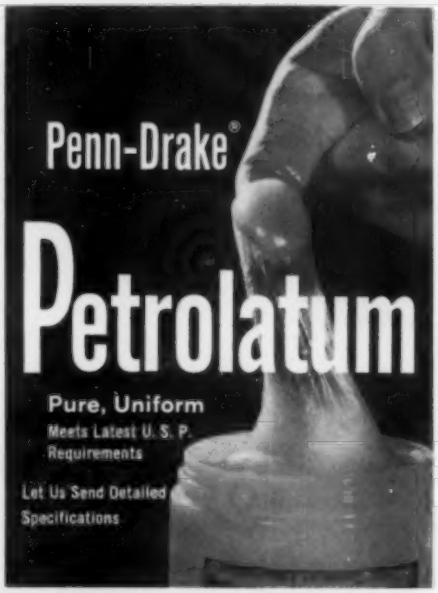

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME 49 *AUGUST 1960* NUMBER 8

Infrared Analysis of Pharmaceuticals I*

Application of the Potassium Bromide Disk Technique to Some Steroids, Alkaloids, Barbiturates, and Other Drugs

By ALMA L. HAYDEN and OSCAR R. SAMMUL

The quantitative potassium bromide disk technique has been investigated and methods have been adopted for its application to some pharmaceuticals. Average deviations from linearity within ±4.0 per cent were obtained with some crystalline compounds. Analyses of pharmaceutical preparations, by hand-grinding or vibrator-grinding methods, resulted in average agreement within ±3 to ±4.5 per cent with ultraviolet spectrophotometric or gravimetric determinations.

THE PUBLICATION in 1952 (23, 26) of the potassium bromide disk method has resulted in investigations into the applicability of this method to compounds of varied origin. There have been reports of successful applications, both qualitatively and quantitatively, to steroids (9, 12, 20, 21), carbohydrates (2, 28), amino acids and peptides (7, 24, 26), pesticides (27), and other organic and inorganic compounds (4–6, 11, 14, 29). From these studies, it has been shown that this method is useful in the study of water-soluble materials and of fractional milligram amounts of substances difficultly soluble in the usable infrared transparent solvents. Another advantage is that some substances which are structurally similar and whose spectra in solution are essentially identical may be differentiated by infrared spectra of their potassium bromide disks (10).

In this laboratory, there developed a need for a method of determining and identifying fractional milligram amounts of pharmaceuticals, some of which were difficultly soluble in the usable infrared solvents. In view of the reports of success with quantitative potassium bromide methods (5, 12, 14, 17, 21, 24, 25, 27, 29), and with an awareness of the possible formation of anomalous spectra (1–3, 8, 10, 18–20), an investigation was made of the applicability of a quantitative potassium bromide method to some pharmaceuticals.

Several methods have been used in obtaining homogeneous potassium bromide-compound mixtures. Kirkland (14) studied various methods of dispersing samples in potassium bromide and concluded that vibrator-grinding gave the most reproducible mixtures. Wiberley and co-workers (29) used vibrator-grinding of potassium bromide-sample with potassium thiocyanate as an internal standard in quantitative studies of poly(vinyl chloride)-poly(vinyl acetate) copolymers. Schwarz and co-workers (25) used lyophilization to obtain homogeneous mixtures of potassium bromide and desoxyribonucleic acid. Rosenkrantz and co-workers (21) estimated to within 8 per cent of the known amount for steriods mixed by spatula with potassium bromide. Susi and Rector (27) used vibrator-grinding of crystalline materials in the quantitative determination of mixtures of pesticides. These authors reported 5 per cent deviations from the Beer-Lambert law

* Received October 30, 1959, from the Division of Pharmaceutical Chemistry, Food and Drug Administration, Washington 25, D. C.

The authors are greatly indebted to Mr. Jonas Carol for his advice and criticism throughout this work.

for standard pesticides. Itō and Amakasu (12) found that trituration in a mortar provided satisfactory preparation of ethinyl estradiol samples, which could be analyzed with an average accuracy of about 2 per cent. Ingebrigston and Smith (11) found that the mixtures prepared by hand-grinding a slurry of the sample and potassium bromide in a volatile solvent gave spectra of excellent quality with increased resolution and absorbance.

In the work reported here, the authors made a preliminary investigation into various methods of dispersing the sample in potassium bromide. It was found that hand-grinding of solutions of compounds with potassium bromide and vibrator-grinding of residues from solution or of recrystallized samples with potassium bromide without an internal standard gave the most reproducible homogeneous mixtures which could be studied quantitatively. Since the crystalline forms of some compounds depend on the solvent and the conditions of crystallization, the standard and sample solutions were subjected to identical treatment.

Some standard steroids, alkaloids, barbiturates, and other compounds were found to agree within ±1.0–4.0 per cent with the Beer-Lambert law over the concentration range of 0.05–0.40 per cent by weight. The results with some cinchona alkaloids are given elsewhere (10).

Application of the described procedures to quantitatively prepared (synthetic) mixtures, to recovery experiments, and to drugs containing hydrocortisone and 17-hydroxy-11-desoxycorticosterone, ethinyl testosterone, phenobarbital, or acetophenetidin and caffeine revealed an average agreement within 3.0 per cent of the added amount or of results obtained by ultraviolet spectrophotometry and within 4.5 per cent of gravimetric measurements.

EXPERIMENTAL

The potassium bromide (Harshaw 200/325) was dried at 105° for a minimum of sixteen hours prior to use. The 20/40 mesh material was prepared in this laboratory from Harshaw random size potassium bromide and was dried as above.

All of the substances studied were purified by recrystallization from a suitable solvent, and their melting points were determined on a Fisher-Johns melting block. For the analytical studies, solvents were chosen from which each of the compounds crystallized without glassy or amorphous material, and solutions of 1.0 mg. per ml. were used.

Methods

Preliminary Sample Dispersion Experiments.—Initial experiments with potassium thiocyanate as an internal standard used lyophilization and hand-grinding as the means of producing the mixtures. For the lyophilization procedure, 5 ml. of a 4% aqueous solution of potassium bromide containing 0.1% potassium thiocyanate was frozen with about 0.5 ml. alcoholic solutions of ethinyl estradiol, hydrocortisone, cortisone, or reserpine while being mechanically rotated. The frozen material was then dried under high vacuum. The dried mixture was mixed briefly with a spatula and a 200-mg. aliquot pressed into a disk. When hand-grinding was employed, an aliquot of a solution containing the sample was ground with 200 mg. of a 0.1% solid mixture of potassium thiocyanate in 200-mesh potassium bromide.

Some early attempts were made to disperse residues from solution with potassium bromide by evaporating the solution to dryness on potassium bromide powder, or by evaporating the solution to dryness in a mortar with subsequent addition of potassium bromide and grinding. When vibrator-grinding of crystalline samples was attempted, it was necessary to weigh small amounts, less than 1 mg., on a microbalance.

Final Hand-Grinding Procedure.—An aliquot of a solution (1.0 mg. per ml.) containing 0.05–1.0 mg. of the compound was ground for an accurately measured and reproduced time (five to ten minutes) with 200 mg. of potassium bromide in a 50-mm. mullite mortar with pestle. The mixture was freed of last traces of solvent and adsorbed water in vacuum at room temperature or at a temperature above the boiling point of the solvent.

Final Vibrator-Grinding Procedure.—A Crescent amalgamator fitted with a steel capsule ($^{5}/_{16}$-inch inside diameter, 1 inch in length) containing three steel balls ($^{1}/_{8}$ inch in diameter) was used for vibrator grinding. An aliquot of a solution containing 0.05–2.0 mg. of the compound was evaporated to dryness with dry nitrogen in the steel capsules containing the steel balls. Standard and sample solutions were evaporated under the same conditions at the same time. The residue was dried at room temperature in vacuum over P_2O_5 for about one hour, 400 mg. of potassium bromide was added, and the cylinder stoppered. The contents were vibrated for a predetermined optimum time (five to twenty minutes).

Disk Pressing Technique.—In general, the potassium bromide mixtures and disks were prepared at room temperature at less than 50% relative humidity to minimize the adsorption of water. Under conditions of greater than 50% relative humidity, the mixtures were dried at 105° at atmospheric pressure, or at an equivalent temperature under high vacuum. The effects of adsorbed water can be reduced by grinding at temperatures higher than atmospheric temperature. For each disk, 200 mg. of potassium bromide-compound mixture was pressed in a Beckman evacuable die which forms disks of 12.7 mm. in diameter and about 0.57 mm. in thickness. After evacuation at less than 1 mm. Hg for one minute, a force of 20,000–25,000 pounds was applied for three minutes during evacuation. After pressing, the disks were placed in vials, heated at 105° for ten to twenty minutes, and cooled to room temperature in a desiccator.

Measurement of Spectra.—The spectra were ob-

tained with a Perkin-Elmer Model 21 double-beam spectrophotometer with sodium chloride optics. The disks were inserted in the sample beam by means of a disk holder which fitted the microcell adapter. The qualitative or quantitative measurements were made without compensation in the reference beam.

A preliminary qualitative spectrum of the 2–15μ region was made of a disk of each compound (about 0.25–0.50%, by weight) which was prepared under moderate hand- or vibrator-grinding conditions (one or two minutes grinding). This spectrum served as a guide in the selection of bands for quantitative study and in the detection of spectral variations.

For quantitative work, the baseline procedure was used to obtain the absorbance using minima on each side of the chosen maximum. After the first absorbance measurement, the disk was turned over in the holder and a second measurement was made. The average was taken of the two baseline absorbance readings. The disk area exposed to the beam was outlined at the time of the first reading and the average thickness was obtained by making six readings of this area with a micrometer. The product of the average thickness and the per cent concentration was considered the effective concentration in the sample beam. A plot of baseline absorbance against effective concentration for a compound revealed the agreement with the Beer-Lambert law over the concentration range studied. For some compounds, two or three bands were chosen from the qualitative spectra and the average of results from these bands was computed.

Prior to a determination of deviation from the Beer-Lambert law, it was necessary to determine the optimum grinding time conditions. Since preliminary work showed that most of these compounds follow the Beer-Lambert law at a concentration of 0.25%, mixtures containing 0.25% of compound were ground for times varying between one and twenty-five minutes by one of the given procedures. By plotting an absorptivity coefficient (baseline absorbance/thickness × concentration) against grinding time for the selected band (s), and by observing the spectrum at the various grinding times, the optimum grinding time was ascertained. For most compounds ten minutes hand-grinding was optimum. The optimum vibration-time varied with the compound, the size of the potassium bromide matrix, and the number of steel balls used.

Using the optimum grinding time, calibration curves showing the relationship between baseline absorbance and effective concentration were made by studying disks of different concentrations at the chosen wavelengths. A concentration which fell on the linear portion of the curve was chosen for analytical purposes.

Analyses of quantitatively prepared (synthetic) mixtures preceded recovery experiments where the standard compounds were used in the various separation procedures given below. The amounts determined by the infrared method were compared with the amounts added or with the amounts calculated from ultraviolet or gravimetric measurements. For most of the experiments, the averages of analyses on duplicate disks were used. The identities of the analyzed compounds were proven by comparison of the spectra of sample and standard disks of 0.10–0.25% by weight.

Analyses of Various Pharmaceutical Preparations

Ethinyl Testosterone.—A weighed amount of ethinyl testosterone tablet mixture equivalent to 25 mg. of active ingredient was chromatographed on a Celite-water column (10 Gm.–5 ml.). The column was washed with 50 ml. isooctane and the excess isooctane blown out with gentle air pressure. The ethinyl testosterone was eluted with 150 ml. chloroform and the eluate evaporated to dryness in vacuum at less than 50° or with a stream of dry air at less than 40°. The residue was dissolved in absolute methanol and diluted to volume in a 25-ml. volumetric flask.

Aliquots (0.10 ml.) of methanolic solutions of the sample and of the standard (1.0 mg. per ml.) were mixed with 200 mg. potassium bromide (200 mesh or 20/40 mesh) by the hand-grinding (ten minutes) or vibrator-grinding (six minutes) procedures. For determinations using the 9.44μ band, aliquots of 0.8 ml. were required. Baseline absorbance measurements were made of the —C=C—C=O band at 6.03μ. The absorptivity coefficient, K_{std}, was calculated for the standard from the equation $K_{std} = A_b/CL$, where A_b is baseline absorbance, C is concentration in per cent by weight, and L is average thickness in millimeters. From the following equation, the calculation of amount of active ingredient in the sample tablet was made.

$$\frac{A_b}{K_{std} \times L_{sample}} \times \text{wt. } KBr \text{ mixture} \times \frac{\text{Total vol.}}{\text{Aliq. vol.}} \times \frac{\text{Av. wt. per tablet}}{\text{wt. of sample}} = \text{mg. per tablet.}$$

Hydrocortisone.—A quantity of tablet mixture equivalent to 20 mg. of hydrocortisone was suspended in dilute acid solution and completely extracted with chloroform. The combined extracts were evaporated to dryness, and the residue was chromatographed on a Celite-formamide-water column. The column was eluted successively with benzene and with chloroform. The benzene and the chloroform eluates were washed with water and were dried with anhydrous sodium sulfate. The dried eluates were filtered, evaporated to dryness, and the residues made to volume with absolute methanol. Aliquots of the methanolic solutions were analyzed using the hand-grinding procedure. The resulting potassium bromide disks were compared quantitatively and qualitatively with standard disks of 17-hydroxy-11-desoxycorticosterone (0.15% by weight) and of hydrocortisone (0.25% by weight) which were prepared at the same time from methanolic solutions of the standard compounds. The quantitative determinations were made of the —C=C—C=O band for each compound (5.99μ 17-hydroxy-11-desoxycorticosterone, 6.05μ hydrocortisone).

Phenobarbital.—A weighed amount of phenobarbital tablet mixture equivalent to 50 mg. active ingredient was dissolved in 5 ml. (2 + 1) formamide-water, and mixed with 5 Gm. acid-washed Celite. This mixture was chromatographed on a modified Sabatino (22) Celite-formamide and water column [5 Gm.–5 ml. (2 + 1)]. A base layer of 1 Gm. acid-washed Celite mixed with 1 ml. saturated barium hydroxide solution was used to remove any stearates

present in the tablet.[1] After a preliminary elution with 150 ml. isooctane-chloroform (1 + 1), phenobarbital was eluted with 200 ml. water-washed chloroform. The chloroform eluate was washed with water, diluted with approximately 2% ethyl alcohol by volume, and evaporated to dryness with dry air at less than 60°. The residue was dried in vacuum over P_2O_5 for thirty minutes, and was made to volume in benzene in a 50-ml. volumetric flask. Aliquots (0.4 ml.) of the sample solution and of a standard solution (1.0 mg. per ml.) were made into disks (0.1% by weight) using the hand-grinding procedure. The quantitative determinations were made using the carbonyl band at 5.88μ.

Phenobarbital in the Presence of Aminophylline.—A weighed portion of sample equivalent to about 50 mg. of phenobarbital was suspended in 50 ml. HCl (1 + 1). The mixture was completely extracted with ether. The combined ether extracts were washed with two 10-ml. portions of HCl (1 + 1), and with water, and were evaporated to near dryness with dry air at less than 35°. The residue was treated as described under "Phenobarbital."

Acetophenetidin and Caffeine in APC Tablets.—The acid-base column described by Levine (15) was modified by using two and one-half times the amounts of column components and twice the amounts of active ingredients, and by incorporating a wash layer consisting of 5 Gm. Celite and 5 ml. distilled water at the top of the column.

An amount of tablet mixture containing about 7.4 mg. acetophenetidin, 1.4 mg. caffeine, and 10 mg. aspirin was chromatographed on the modified column. The acetophenetidin was eluted with ether, the eluate was evaporated to dryness, and made to volume (5 ml.) in absolute methanol. The caffeine was eluted with water-washed chloroform, the eluate was diluted with 5% ethyl alcohol by volume, evaporated to dryness, and made to volume (2 ml.) in benzene. The aspirin was not determined.

Solutions of standard acetophenetidin in methanol (1.48 mg. per ml.) and of standard caffeine in benzene (0.72 mg. per ml.) were prepared from accurately weighed crystalline samples. Residues of aliquots (1 ml.) of the standard and sample solutions of acetophenetidin and of caffeine were mixed, separately, with 400 mg. of 200/325 mesh and 20/40 mesh of potassium bromide for twenty and five minutes, respectively, using the vibrator-grinding procedure. Quantitative determinations were made using baseline absorbances of the bands at 8.52, 9.54, and 11.94μ for acetophenetidin, and at 13.19μ for caffeine.

RESULTS AND DISCUSSION

In the authors' hands, the method of lyophilization proved unsuccessful as a means of dispersing samples in potassium bromide for quantitative studies. However, qualitative spectra of mixtures prepared in this way were essentially the same as those obtained using the two adopted procedures.

In preliminary experiments with potassium thiocyanate as an internal standard, disks of dispersions of this compound with ethinyl estradiol, hydrocortisone, and reserpine and potassium bromide revealed the C ≡ N band at 4.87μ. The spectra of these compounds differed from those in potassium bromide disks alone by the presence of this band. However, in disks of potassium thiocyanate with cortisone and potassium bromide, two bands of varying intensities appeared in the C ≡ N region at 4.81 and 4.87μ. In addition, on heating disks of this mixture the 5.98μ band attributed to the —C=C—C=O group of cortisone and the 4.87μ band were seen to disappear. Extraction of these heated disks with chloroform and reformation of a potassium bromide disk from the extract residue revealed the normal spectrum of cortisone with the C=O band at 5.84μ and the —C=C—C=O band at 5.98μ. These observations are indicative of bonding or complex formation between the —C=C—C=O group of cortisone and the —C≡N group of potassium thiocyanate. Because of these variations, the difficulty of obtaining reproducible potassium bromide-potassium thiocyanate mixtures, and the ease of making accurate thickness measurements, the use of an internal standard was avoided.

Mixtures which were produced by hand-grinding crystalline samples and residues or by vibrator-grinding weighed residues with potassium bromide were not as quantitatively reproducible as, or required more time than, those prepared by the final methods.

In general, under the described experimental conditions, disks of hand-ground mixtures gave spectra with somewhat better resolution and more intense absorbance than disks of vibrator-ground mixtures. In Fig. 1 are seen qualitative spectra of the 2–15μ region of some of the compounds studied. Although vibrator-grinding is mechanically more efficient, because of the grinding force exerted on the crystal, it is more likely to produce mixtures whose disks show spectral changes and distortions. Under conditions of greater than 50% relative humidity, the vibrator-grinding procedure suffers from inadequate mixing as a result of adsorption of moisture from the atmosphere. In addition, hand-grinding is preferable for those compounds which exhibit spectral changes on vibrator-grinding. When the hand-grinding procedure is accurately reproduced, the results are as reliable as those where vibrator-grinding is employed.

In general, the effects of particle size of the matrix on the ease of disk formation and on the qualitative spectrum agree with previous reports (1, 16). It was found that samples mixed with the coarse 20/40 mesh potassium bromide required less grinding, adsorbed less water, and produced clearer disks than did the 200/325 mesh powder. Vibrator-grinding of 200/325 or 20/40 mesh potassium bromide for longer than twenty minutes produced a powder of such small particle size that disk formation was difficult in that the disks were flaked and cracked when pressed from the die. With the vibrator-grinding procedure, those compounds which showed a tendency for spectral changes exhibited these changes with less grinding time with the 20/40 mesh crystals than when the 200/325 mesh powder was used. It is presumed that the larger particles of potassium bromide contribute to the grinding of the sample. As a result, anomalies may occur more readily than when the finer particles of potassium bromide are used.

[1] In recent experiments, the $Ba(OH)_2$ layer has been omitted with an increase in the reproducibility of recoveries.

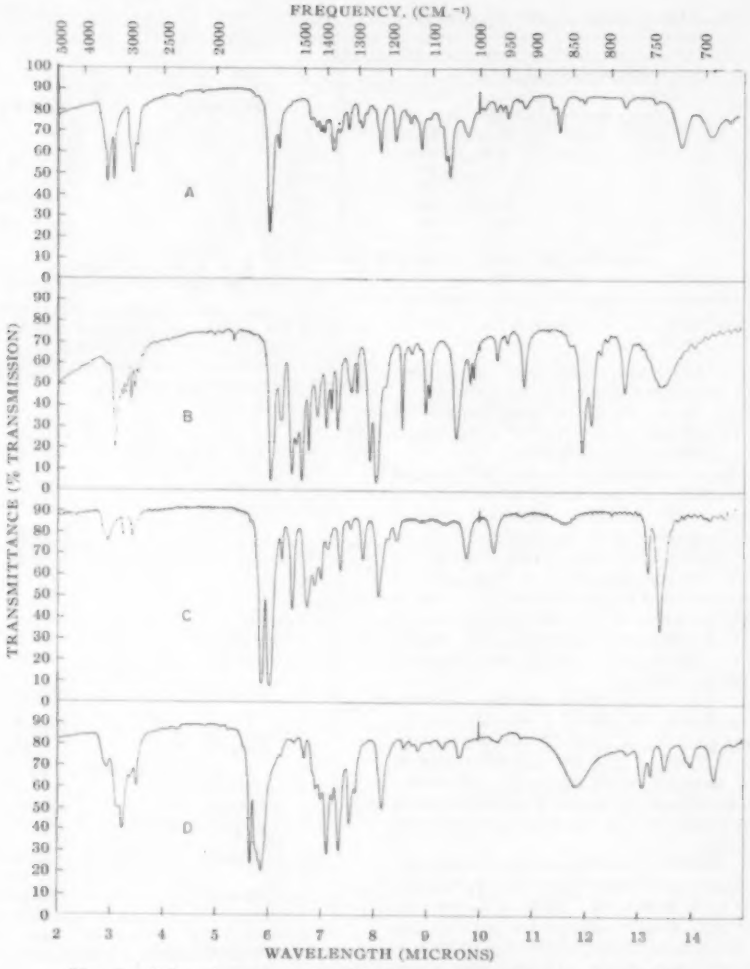

Fig. 1.—Infrared spectra of ethinyl testosterone from methanol (*A*), acetophenetidin from methanol (*B*), caffeine from benzene (*C*), and phenobarbital from benzene (*D*) in potassium bromide disks.

The clarity of the disks was a function of the amount of sample present, the size of the potassium bromide matrix, and the extent of grinding. Some compounds formed clear disks at concentrations up to 0.5 mg. per 200 mg. potassium bromide; disks of other compounds were cloudy at concentrations of about 0.2 mg. per 200 mg. potassium bromide. Cloudy disks could be used with satisfactory results as the baseline absorbance measurement eliminated the effect of background variation. Variable results were obtained when cracked or striated disks were compared with clear disks.

A potential source of absorbance measurement error is the presence of interference patterns in the spectrum. When these fringes were encountered, the baseline was drawn so as to bisect the highest absorbing areas of the minima on each side of the band maximum.

A study of the changes in spectrum with grinding time and concentration reveals the stability of the compound under the grinding procedure. In Table I are given the absorptivity coefficients at different grinding times for some of the compounds studied. The optimum grinding time was chosen as the time producing maximum absorbance of the selected band and maximum spectral resolution with identity with the qualitative spectrum of the compound.

The authors' investigations indicated that the stability of a compound to the grinding conditions is a function of the crystal energy of the compound and of the stability of the disarranged crystalline lattice under the experimental conditions. Acetophe-

TABLE 1.—CHANGES IN ABSORPTIVITY COEFFICIENT WITH GRINDING TIME

	Absorptivity Coefficient			
Minutes	Ethinyl Testosterone, 6.03μ	Amobarbital, 8.07μ	Acetophenetidin, 11.94μ	Caffeine, 13.19μ
1	..	..	..	69
3	720	107	..	96
5	730	..	256	105
10	765	134	256	98
15	770	143	274	98
20	..	136	287	..
25	..	137	272	..

netidin (m.p. 135°) and amobarbital (m. p. 156–158.5°) did not exhibit spectral changes; however, phenobarbital (m.p. 174–178°), quinidine (m. p. 172–173°), and quinine (m. p. 174–175°) (10), showed variations in spectra with grinding time.

The results of quantitative studies on standard ethinyl estradiol, hydrocortisone, 17-hydroxy-11-desoxycorticosterone, cortisone, reserpine, ethinyl testosterone, ascorbic acid, amobarbital, phenobarbital, acetophenetidin, and caffeine revealed the average deviation from linearity to be within ±4.0% over the concentration range 0.05–0.40% by weight. The smallest variation of 1% was seen for ethinyl estradiol, hydrocortisone, cortisone, and ethinyl testosterone. The largest variation was seen with ascorbic acid and caffeine.

In Figs. 2–4 are given the relationship between baseline absorbance and effective concentration for some of the compounds studied. In general, the absorbance, resolution, and the reproducibility of the spectra were improved by heating the disks. It is seen that the selected bands of acetophenetidin and caffeine follow the Beer-Lambert law within the described limits.

The 6.03μ band of ethinyl testosterone shows linearity up to 0.065–0.10% by weight when the minima at 5.45 and 6.65μ were used. Disks of higher concentrations vary from a straight line through the origin but show a straight line relationship with intercept at about 0.1 absorbance. In later work it was found that an improved relationship is obtained for the higher concentrations if band-broadening effects are considered. When the product of baseline absorbance times the apparent band width (in cm.) at one-half the height of absorbance is plotted against effective concentration (Fig. 2), the 6.03μ band follows the Beer-Lambert law within ±2% for concentrations up to 0.25% by weight. Concentrations below or above 0.065% can be analyzed using baseline absorbance alone if the standard is of the same concentration. The 9.44μ band showed deviations of ±4.0% at concentrations up to 0.25% by weight.

For a given compound, all bands did not follow the Beer-Lambert law. In addition, for some compounds (caffeine and phenobarbital), bands which obeyed the Beer-Lambert law on direct determination gave nonlinear results when the standard compounds were carried through the separation procedures. Only those bands which showed linearity after the compound was subjected to the separation procedure were used.

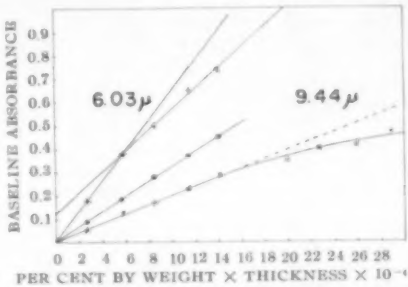

Fig. 2.—Calibration curves of ethinyl testosterone showing the relationship between baseline absorbance and effective concentration (×), and between baseline absorbance times apparent half-band width and effective concentration (⊙).

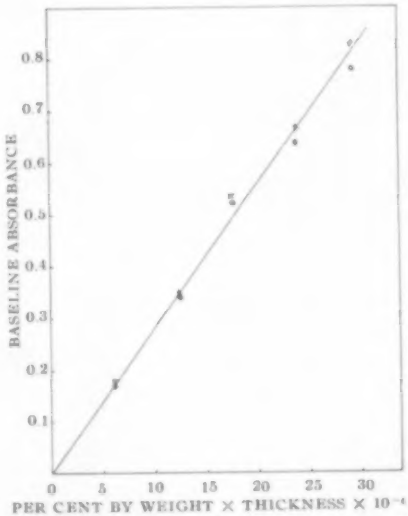

Fig. 3—Calibration data for acetophenetidin (11.94μ) before (o) and after (×) heating potassium bromide disks.

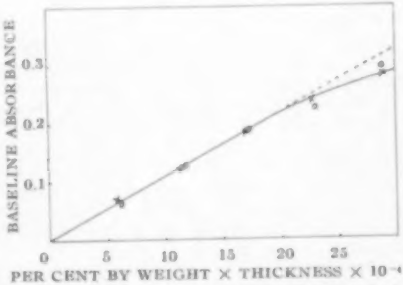

Fig. 4.—Calibration data for caffeine (13.19 μ) before (o) and after (×) heating potassium bromide disks.

TABLE II.—*K* VALUES

Experiment No.	Ethinyl Testosterone, 6.03μ Hand-Ground/Vibrator-Ground		Phenobarbital, 5.88μ Hand-Ground	Acetophenetidin[a] 9.54μ	Acetophenetidin[a] 11.94μ	Caffeine[a] 13.19μ
1	762, 762	664, 660	620	..	..	105
2	746, 760	640	614, 613	249, 256	261, 274	95, 105
3	758, 761	673	..	246	272	109.5
4	763, 761	..	..	..	..	..

[a] Vibrator-ground.

TABLE III.—TABLE OF RECOVERIES FOR ETHINYL TESTOSTERONE

Sample	Added or Declared mg./Tablet	Found per Tablet[a] Ultraviolet mg.	Ultraviolet %	Infrared Hand-Ground mg.	Hand-Ground %	Infrared Vibrator-Ground mg.	Vibrator-Ground %
Standard	4.85	4.81	99.1	4.75	98.0	..	..
Standard	25	..	..	24.85	99.4	..	..
A	25	26.90	107.5	27.20	108.8	27.00	108.0
B	10	9.53	95.3	9.65	96.5	9.53	95.3
C	25	24.70	98.8	24.85	99.4	25.25	101.0
D	10	9.48	94.8	9.54	95.4	9.38	93.8

[a] Values represent the average of at least two determinations.

TABLE IV.—ANALYSES OF SOME PHARMACEUTICAL COMPOUNDS AND PREPARATIONS[a]

Active Ingredient	Sample Description	Added mg. or Declared mg./Tablet	Found Ultraviolet or Gravimetric, mg.	%	Infrared mg.	%
Hydrocortisone	Standard	2.02	..	..	2.00	99.0
Hydrocortisone + 17-hydroxy-11-desoxycorticosterone	Tablets	20.0	8.6[b]	43.0	9.5	47.5
			11.2[b]	56.0	10.5	52.5
Phenobarbital	Standard	50.0	48.1	96.2	47.3	94.6
Phenobarbital	Tablets	97.2	94.4	97.1	94.8	97.5
Phenobarbital + aminophylline	Tablets	32.4	31.6	97.5	32.1	99.0
Acetophenetidin	Standard	37.0	37.1	100.2	37.4	101.1
Acetophenetidin + acetanilid	Synthetic mix	1.0 0.1	..	..	0.994	99.4
Acetophenetidin + acetanilid	Synthetic mix	1.0 0.5	..	..	0.990	99.0
Acetophenetidin	Synthetic mix	37.0	38.6	104.3	38.5	104.1
Acetophenetidin	Tablets	162.2	146.5	90.3	147.0	90.6
Caffeine	Standard	7.34	7.27	99.0	6.9–7.56	94–103
Caffeine	Synthetic mix	7.36	7.84	106.5	7.75	105.2
Caffeine	Tablets	32.4	28.6	88.1	29.5	91.0

[a] These results are averages of two or more determinations. [b] Gravimetric.

In Table II are given some of the *K* values for the selected bands obtained from ethinyl testosterone, phenobarbital, acetophenetidin, and caffeine which were carried through the given procedures. It is seen that, except for caffeine, the values agree within 3% with the average values. A maximum of 8% variation from the average was shown by caffeine. The average *K* values of duplicate disks of the standard compounds were used in the determination of the corresponding pharmaceutical preparations.

The results which are given in Tables III and IV were corrected for the recoveries of the standards from the described procedures. In Table III are seen the results of ultraviolet and infrared determinations of ethinyl testosterone from recovery experiments and from analyses of tablets using the described procedure. On the basis of ultraviolet and infrared results, a 99% recovery was obtained of this compound from the procedure. The analyses of several tablets reveal excellent agreement (within 1–2%) between ultraviolet and infrared data.

From Table IV, it is seen that there is a 3.5–4.5% variation between the infrared and gravimetric determinations of hydrocortisone and of 17-hydroxy-11-desoxycorticosterone. Acetophenetidin, as determined by infrared, agreed within 1% with those results determined by ultraviolet spectrophotometry. Analyses of quantitative mixtures of this compound with acetanilid showed 99% of the amount added. A 100% recovery was obtained when standard acetophenetidin was carried through the separation procedure.

The greatest disparity was seen between the ultraviolet and infrared determinations of caffeine. Although a 99% recovery of standard caffeine was obtained by ultraviolet determinations, the recovery

as indicated by infrared determinations varied between 94 and 103%. As shown in Table IV, when an average K value is used, the infrared results from the analyses of a synthetic mixture agree within 1.2% with ultraviolet results. The analyses of a tablet mixture revealed an average variation of 3.0%. The fact that this compound became slightly discolored on evaporation to dryness after the separation may explain the larger variations.

Phenobarbital exhibited three spectra when attempts were made to apply the vibrator-grinding procedure. Because of this, the hand-grinding procedure was used in the analysis of this compound. Under the described procedure, a single reproducible spectrum was obtained. Comparison of the 5.88μ carbonyl band of standard and sample carried through the same procedure gave results which agreed within 1% of the ultraviolet results. In the preparation containing aminophylline, there was a 1.5% difference between ultraviolet and infrared results. The recovery of standard phenobarbital from the column was 96% of the amount added.

Attempts to analyze reserpine and ascorbic acid in dosage forms were unsuccessful because of the instability of these compounds.

The effect of crystalline form on the infrared spectra of solids has been emphasized (1, 6, 20). Since the relationship between structure and vibration spectrum of a crystalline compound is based on the unit cell of the crystal and not on the individual molecules (13), it is apparent that the standard and sample must have the same crystalline form in order to produce identical infrared spectra. The methods described here insure the use of identical crystalline forms for standard and unknown by subjecting them to identical treatment. The inclusion of the steps for removing final traces of solvent and adsorbed water from the potassium bromide-compound mixture eliminates the effects of solvent bands on the spectrum.

The results show that the quantitative potassium bromide method can be used in the analysis of compounds of varied types. The agreement between the added amounts and the amounts detected when standards were subjected to the separation procedures is evidence of the excellent recovery for most of the compounds. The agreement between ultraviolet and infrared results on pharmaceutical preparations attests to the usefulness of this procedure. Provided anomalous spectra are not encountered, the results from analyses of potassium bromide disks can provide both quantitative determination and qualitative identification.

Because of the time involved in preliminary investigation, it is thought that this method will be most widely applicable and acceptable for the analyses of polar and isomeric compounds which cannot be analyzed more easily by combining more conventional means of determination with identification by infrared spectroscopy.

SUMMARY

These experiments show that the quantitative potassium bromide method can be applied in the analyses of compounds of varied origin. In general, the results agree within 1 to 4 per cent with the amount added or detected by other means in synthetic mixtures and in recovery experiments. Comparison of standard and sample subjected to the same separation procedure, the same solvent, and the same grinding regimen give infrared results which agree within 3 per cent with those obtained from more conventional methods of analysis of pharmaceutical preparations.

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Infrared Analysis of Pharmaceuticals II*

A Study of the Cinchona Alkaloids in Potassium Bromide Disks

By ALMA L. HAYDEN and OSCAR R. SAMMUL

In the course of an investigation into the applicability of a quantitative potassium bromide disk method to various drugs and chemicals, the cinchona alkaloids, quinidine, quinine, cinchonine, and cinchonidine were studied. Under certain experimental conditions, possibly due to the formation of dimorphous and amorphous forms, anomalous spectra were obtained for quinidine and quinine. Cinchonine and cinchonidine did not exhibit significant variations in spectra under the conditions investigated.

BECAUSE of the similarity in structure between quinidine and quinine, and between cinchonine and cinchonidine, and because of rotational isomerism, the infrared spectra of the two isomers of each series in carbon disulfide or chloroform solutions (Figs. 1 and 2) are very similar. As a result, these spectra are not easily differentiated, nor do they afford unequivocable quantitative determination when the presence of the isomer is possible.

The publication in 1952 (13, 14) of the potassium bromide disk method has resulted in successful applications to compounds of varied origin. However, there have been several reports (1–3, 6, 10–12) of anomalous spectra obtained with this method. In general, the origins of these anomalies may be placed in four groups: (*a*) formation of polymorphic crystalline forms, (*b*) formation of amorphous materials, (*c*) formation of hydrates, and (*d*) chemical transformations or combinations.

When an attempt was made to apply a quantitative potassium bromide method (7) to four cinchona alkaloids, anomalies of groups (*a*) and (*b*) were indicated with quinidine and quinine. Cinchonine and cinchonidine were stable under the conditions studied.

EXPERIMENTAL

Quinidine was purified by formation of the tartrate and the hydroiodide, followed by neutralization, and recrystallization from benzene. Quinine was obtained by formation of the oxalate from the recrystallized hydrochloride, with subsequent neutralization with ammonium hydroxide, and recrystallization from benzene. Cinchonine was purified by the mercuric acetate process (15), and was recrystallized from boiling alcohol or from benzene. Cinchonidine was purified by formation of the tartrate and subsequent neutralization. Recrystallization was effected from one part alkaloid in thirty parts benzene or in six parts alcohol. The recrystallized alkaloids were dried at 105° under high vacuum. Their melting points (uncorrected) on a Fisher-Johns melting block were: quinidine, 172–173°; quinine, 174–175.5°; cinchonidine, 206–207°; and cinchonine sublimed with decomposition, about 245°. In a sealed tube, cinchonine melts at 255°.

The spectra were obtained with a Perkin-Elmer model 21 double-beam spectrophotometer with sodium chloride optics. The spectra of the carbon disulfide solutions (less than 3 mg. per ml.) were determined using a 3-mm. microcell compensated with a variable space cell. The spectra of the chloroform solutions (3–10 mg. per ml.) were determined with 1-mm. macrocells.

In general, the potassium bromide mixtures and disks for qualitative and quantitative spectra were prepared as described (7) from aliquots of a benzene or methyl alcohol solution (1.0 mg. per ml.). For quantitative determinations, hand-grinding of solutions for ten minutes or vibrator-grinding of residues, which had been heated at 105°, for five minutes was employed. The resulting potassium bromide mixtures and disks were heated at 105° and the spectra were measured.

RESULTS AND DISCUSSION

When the four recrystallized alkaloids were dispersed in potassium bromide under moderate conditions (7), their infrared spectra (Fig. 3) were distinctly different. Disks of cinchonine and cinchonidine (Fig. 4) followed the Beer-Lambert law within ±3.0% up to a concentration of 0.4% by weight at 13.08 and 13.19μ, respectively.

Quinidine, recrystallized from benzene and dried at 105° in vacuum, gave spectrum *A* of Fig. 5 when 1 mg. was ground for one or two minutes with 200 mg. of potassium bromide. Spectrum *B* was obtained on vibrator- or hand-grinding this mixture for five or ten minutes, respectively. In addition spectrum *B* was obtained when quinidine was vibrator-ground, and the fused product was dispersed in potassium bromide under moderate conditions. On microscopic examination, this fused product appeared to be a mixture of crystals and amorphous material.

Continued grinding of form *B* resulted in partial reversion to form *A*. The rates of the transformations varied with the amounts of starting materials, the grinding time, and the force applied by hand or by the steel balls in the vibrator. In Fig. 6 are

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The crystallographic determinations by Arnold Schulze are gratefully acknowledged.

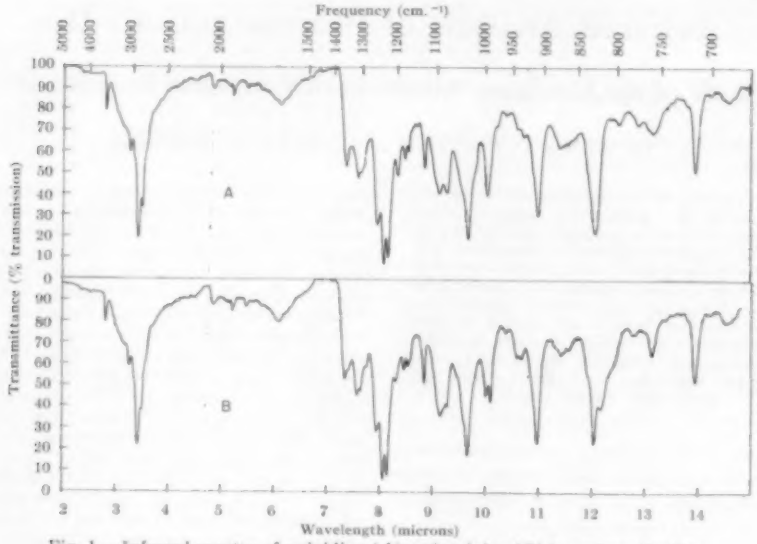

Fig. 1.—Infrared spectra of quinidine (*A*) and quinine (*B*) in carbon disulfide.

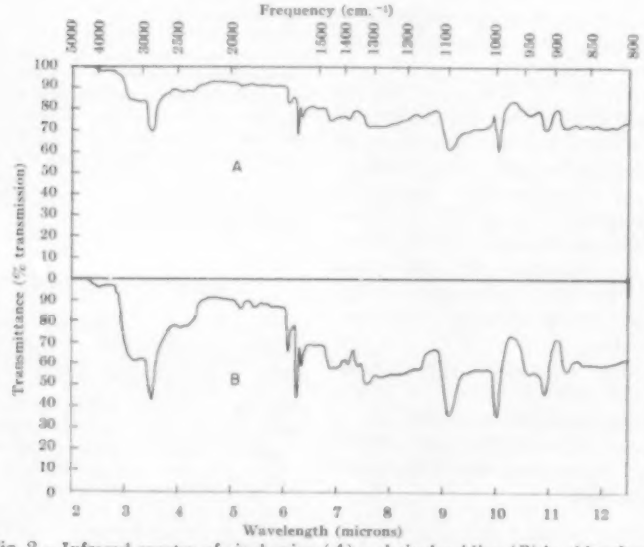

Fig. 2.—Infrared spectra of cinchonine (*A*) and cinchonidine (*B*) in chloroform.

shown some changes in spectrum in the 6–9μ region with grinding time.

There have been reports of differences in the spectra of polymorphic forms of various compounds (4, 5, 9). Chapman (4) obtained the lowest melting forms of certain glycerides by quenching the liquids to 0°. Higher melting forms were detected by heating the quenched material or by cooling the liquid, while rapidly scanning parts of the infrared spectrum.

When quinidine was melted under anhydrous conditions under vacuum and the melt was quenched at 0° to −2°, the resulting glassy material was faintly yellow and gave spectrum *B* of Fig. 5. On a Fisher-Johns melting block, under 10× magnification, the quenched quinidine showed a transition point with partial melting at 74–76°, resolidification and gradual opaqueness above 85°, and final melting at 171–172°. The transition at 74–76° was not as apparent for the ground quinidine. However, spectrum *A* of Fig. 5 was obtained when this material was heated

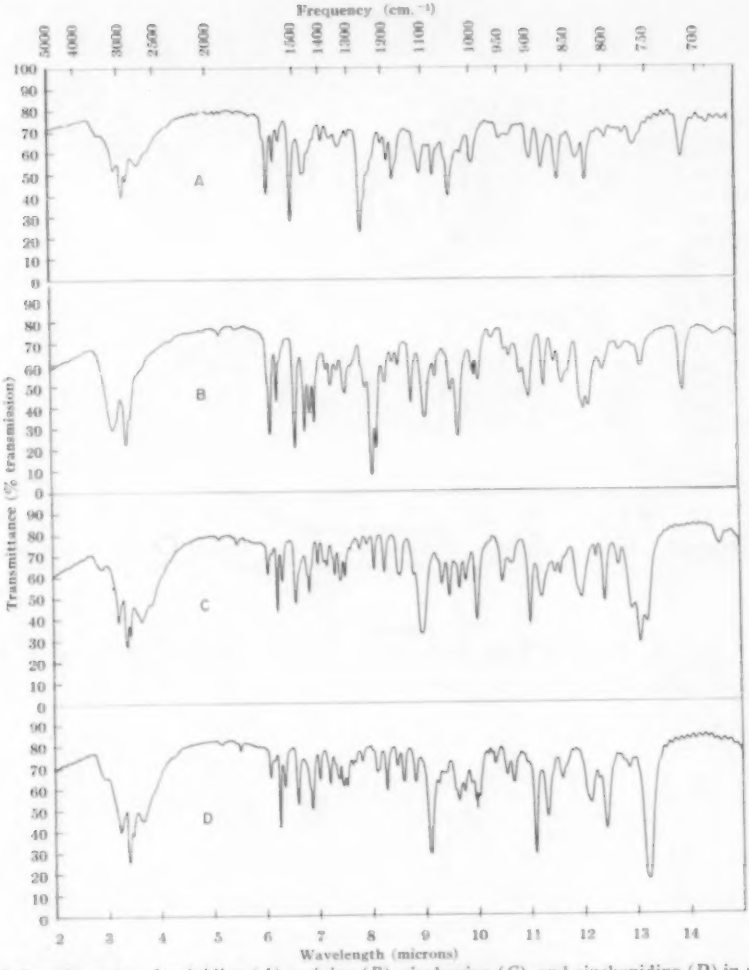

Fig. 3.—Infrared spectra of quinidine (*A*), quinine (*B*), cinchonine (*C*), and cinchonidine (*D*) in potassium bromide disks.

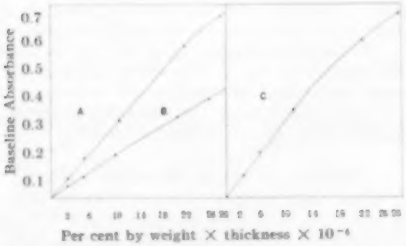

Fig. 4.—Calibration curves of cinchonidine at 13.19μ (*A*), cinchonine at 13.08μ (*B*), and quinine at 8.02μ (*C*) in potassium bromide disks.

at that temperature for thirty minutes. The ground quinidine became opaque above 85° and melted at 170–171°. Spectrum *A* was obtained from both final melting materials and from disks of form *B* which were heated at 75° or above.

Presumably, form *B* is produced as a result of localized heating and cooling during the grinding process. As suggested by Roberts (12), it is possible that as the sample is subjected to frictional forces during grinding, the local temperature is raised above the melting point of the compound. As these forces are removed from the crystals, immediate cooling might effect a rearranged form which is stable up to 75°. At higher temperatures, rearrangement to the crystal lattice of the higher-melting form could occur. This view is supported by the experiments with the quenched melt, by the fused condition of quinidine when ground under extreme conditions, and by the transition at 74–76°.

The spectrum of form *B* is somewhat similar to

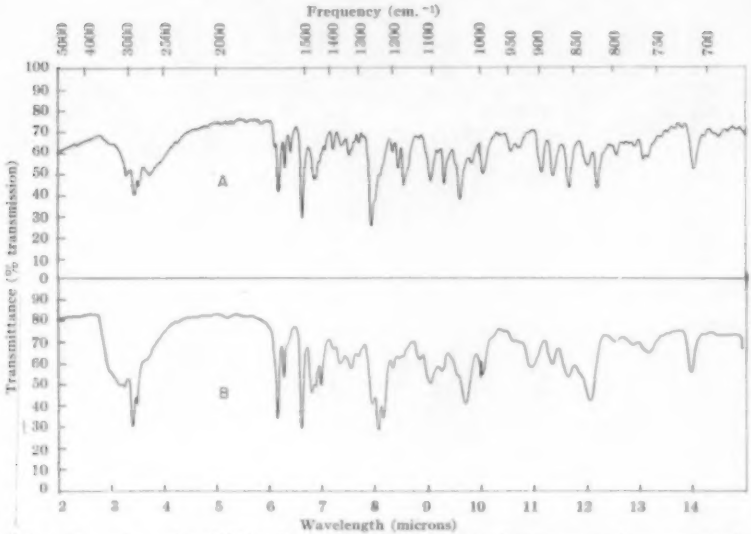

Fig. 5.—Infrared spectra of quinidine hand-ground (*A*), and vibrator-ground or quenched melt (*B*) in potassium bromide disks.

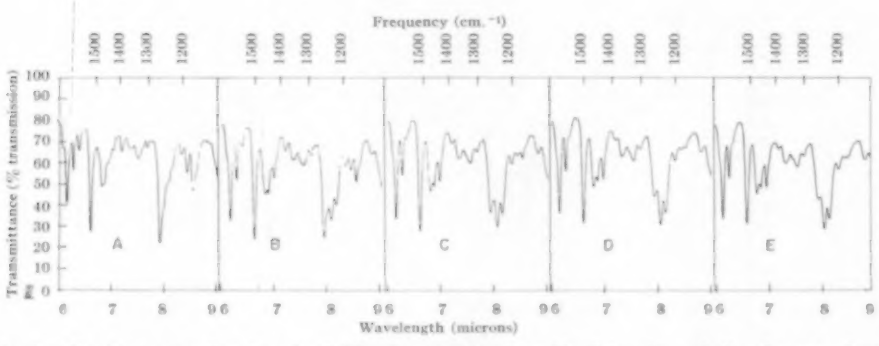

Fig. 6.—The 6–9μ spectral region of quinidine in potassium bromide disks after grinding for one minute (*A*), fifteen minutes (*B*), thirty minutes (*C*), forty-five minutes (*D*), and after melting and quenching (*E*).

those of quinidine recrystallized from 95% ethyl alcohol and from water (Fig. 7). Therefore, the similarity of its spectrum to those of disks of quinine and of quinidine and quinine in solution is not construed as the result of amorphous material alone in the ground or quenched quinidine. When the crystalline solvates were dried at 105° in vacuum, there was a loss in weight equivalent to 1 mole ethyl alcohol and 1½ moles of water, respectively, and reversion to form *A*. Crystallographic examinations of these compounds were incomplete; only the α and γ refractive indexes were determined. Although the α or γ refractive index of the solvates and those of form *A* were different the indexes of form *B* were similar to those of form *A*.

Hydrate formation, as an explanation of spectrum *B* of quinidine, was ruled out when the ground or quenched material was dried at 105° under vacuum for three hours with reversion to form *A* without loss in weight. Compound decomposition or structural rearrangement is unlikely because of the ease of conversion from one form to the other. In addition, the appearance of form *B* on melting and quenching, the final melting point of the reverted quinidine, and the optical rotation values shown in Table I, make the possibility of significant decomposition or rearrangement remote.

TABLE I.—OPTICAL ROTATION VALUES

Sample	$(\alpha)_D$ in Absolute Ethanol, 24°C.
Quinidine	+258°
Quinidine grind	+253°
Quinidine melt	+257°
Quinidine melt heated	+254°
Quinidine . EtOH	+253°
Quinidine . 1½ H_2O	+259°

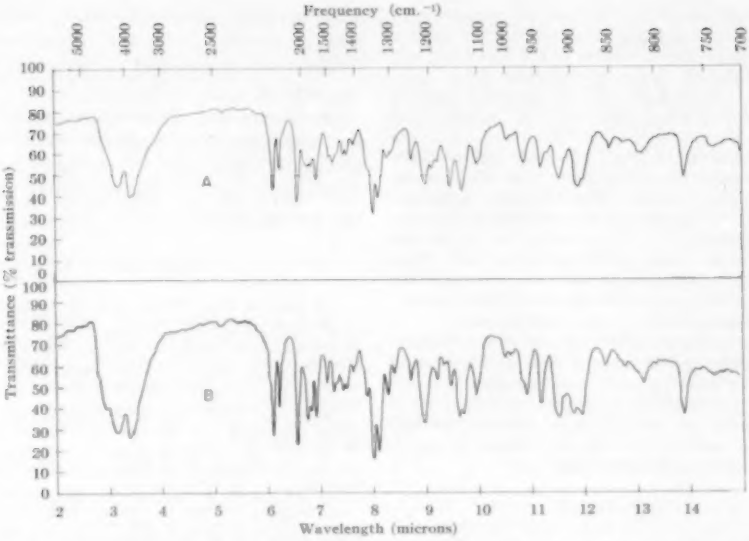

Fig. 7.—Infrared spectra of quinidine recrystallized from 95% ethyl alcohol (*A*), and from water (*B*) in potassium bromide disks.

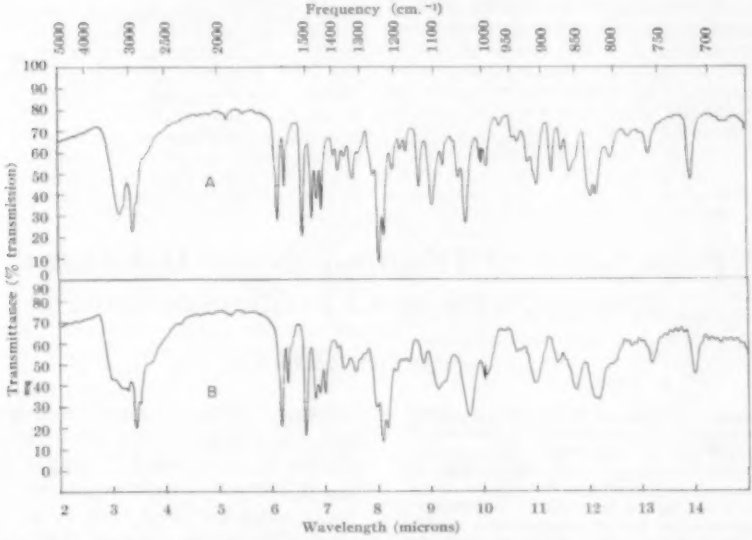

Fig. 8.—Infrared spectra of quinine moderately ground (*A*), and vigorously ground or melted and quenched (*B*) in potassium bromide disks.

Although the presence of dimorphous material in form *B* is indicated, an unequivocable assignment must await additional information, including complete crystallographic data. However, as the relationship between the structure and vibration spectrum of a crystalline compound is based on the unit cell of the crystal (8), the observed differences between the two spectra of quinidine are understandable.

Somewhat less spectral variations were encountered with disks of quinine (Fig. 8). The grind and the quenched melt were obtained under conditions similar to those described for quinidine. However, the transformations were controlled by hand-grinding and by heating disks of quinine at temperatures above 75°. The relationship between absorbance at 8.02μ and effective concentration is shown in Fig. 4.

It has been suggested (1) that the occurrence of polymorphism with the potassium bromide method is related to the crystal energy of the compound and to the stability of the polymorphic form. Significantly, the spectrum of the quenched melt of cinchonine is identical with that of the recrystallized alkaloid. Except for increased absorbance at 10.92μ at the expense of the 11.05μ band, the spectra of the quenched melt of cinchonidine and the starting material were the same. There were no significant changes in the spectra of these two alkaloids after vibrator-grinding for periods up to forty-five minutes. The stability of acetophenetidin (m.p. 135°) and amobarbital (m. p. 158°) (7), under the given experimental conditions, reveals that spectral variations are not dependent on melting point.

Some attempts to control the conversion of quinidine by reducing the grinding time and force, and by vibrator-grinding at 80° were not quantitatively reproducible. Work is in progress on a method of determination by ultraviolet or infrared spectrophotometry and identification by infrared spectroscopy of potassium bromide disks.

SUMMARY

Dimorphous and amorphous forms of quinidine and quinine are indicated when these compounds are ground with or without potassium bromide. By heating quinine-potassium bromide mixtures and the resulting disks, a linear relationship between absorbance and concentration times thickness is obtained. Quantitative determinations of quinidine in potassium bromide are variable.

Cinchonine and cinchonidine do not exhibit significant spectral variations under the experimental conditions. Their disks follow the Beer-Lambert law at concentrations up to 0.4 per cent by weight.

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Preparation of Tritium-Labeled Halothane (2-Bromo-2-chloro-1,1,1-trifluoroethane)*

By CALVIN HANNA

Halothane was labeled with tritium (Wilzbach method) by exposure to 2.3 curies of tritium gas for fourteen days. The one hydrogen of the molecule was labeled and on purification gave a final specific activity of 2.9 mc. per Gm. In the preparation a considerable amount of 'loose' tritium and highly active impurities were separated and the purity of the final product following gas chromatographic purification was better than 99.95 per cent.

HALOTHANE[1] (CF_3-CHBrCl) is receiving attention as a new general anesthetic agent (1). This compound gives fast inductions and recoveries and has been used safely in over five thousand patients (2). Due to the anesthetic potency of this material the inhaled concentration should not exceed two per cent (2) which means rather low blood levels for an anesthetic agent. Due to the chemical stability of halothane the chemical procedure for its determination in blood and tissues is difficult and time consuming (3). An alternate approach to this problem is to use isotopically-labeled halothane as a tracer in designing and testing new methods for its determination. Tritium-labeled halothane was prepared by the Wilzbach method (4) and this report covers the purification and determination of the chemical purity of the final labeled product.

* Received November 27, 1959, from the University of Vermont, College of Medicine, Burlington.
This investigation was supported by a senior research fellowship, SF-156, from the Public Health Service.
[1] Fluothane is the registered trade name of halothane of Ayerst Laboratories, Inc., New York, N. Y.

PROCEDURES

Tritiation.—Two and one-half milliliters of halothane (Fluothane, Ayerst Laboratories, Inc., New York, N. Y., containing 0.01% thymol and less than 0.005% impurities) was sealed in a 20-ml. round-bottom flask (fitted with a break seal), with 2.3 curies of tritium gas at 27° and 0.39 atm. for fourteen days. The flask was cooled in liquid nitrogen, opened and the excess tritium gas pumped off. The remaining radioactivity was 85 mc. of tritium per ml. of halothane.

Purification.—The flask was resealed and a ground-glass joint fused to the open end of the break seal. A long condenser was attached and 20 ml. of ice cold water was poured into the condenser. Halothane is very volatile and this heavy liquid was kept under water in the cold. After the flask was cooled the break seal was smashed with a long glass rod through the condenser. The contents of the flask were shaken for one hour and a sample of the organic layer was assayed, giving an activity of 18 mc. per ml., with the remaining activity being in the aqueous layer.

For purification a 1-ml. aliquot of the organic layer was mixed with 9 ml. of nonlabeled halothane under 10 ml. of cold water. After extracting the organic layer with four additional 10-ml. aliquots of water in order to remove any remaining loose tritium, the organic layer was again assayed, giving a specific activity of the diluted material of 1.4 mc. per ml. Next the organic layer was washed with 10-ml. aliquots of dilute hydrochloric acid, dilute sodium hydroxide, and finally with water. The organic layer was then distilled from 20 ml. of water, giving an activity of 1.0 mc. per ml. A sample of this was subjected to gas chromatographic separation and analysis. The organic layer was fractionated by one pass through a dinonyl phthalate column (stage 1) and then by two passes (stages 2 and 3) through a polyethylene glycol column. The results were as follows:

(*a*) Stage 1, 'light' fraction (at 2% of the total volume) containing halothane with normal halothane impurities.

(*b*) Stage 1, 'heavy' fraction (at 1% of the total volume) containing halothane with any impurities of longer retention time. The main fraction in stage 1 was refractionated.

(*c*) Stage 2, 'heavy' fraction (at 10% of the total volume) containing water, impurities, and halothane. The main fraction from stage 2 was refractionated.

(*d*) Stage 3, 'heavy' fraction (at 2% of the total volume) containing traces of halothane, impurities, and water.

(*e*) Stage 3, 'main' fraction (at 85% of the total volume) containing chromatographically pure (less than 0.005% impurity) halothane.

Of the total radioactivity in the fractioned sample (*a*) had 29%, (*b*) 14%, (*c*) 2%, (*d*) trace, and (*e*) the main fraction 55%. The specific activity of the original halothane before dilution was 2.9 mc./Gm. The purity of the diluted (1:10 v/v) halothane was better than 99.995%, or the purity of the original product was better than 99.95%.

Counting.—Halothane-tritium was counted in a liquid scintillation counter (Packard Tri-Carb) using toluene-tritium as the internal standard. The counting solution was composed of 4 Gm./L. of DPO (2,5-diphenyloxazole) and 100 mg./L. of POPOP [1,4-di(5-phenyloxazolyl)benzene] in toluene.

DISCUSSION

Some of the original activity could have been due in part to the labeling of the thymol 0.01% and traces of normal halothane impurities of less than 0.005%. The thymol should have been removed during the repeated washings and on distillation. Some halothane was lost with the repeated washings since the solubility at 20° in water is 3.45 mg./ml. To reduce this loss the extractions were carried out in the cold at near 0° and in the final step excess water was separated mechanically by freezing in a dry ice-acetone mixture with the organic layer being decanted from the ice crystals. The vapor pressure of this liquid presents problems in purification since it is quite volatile and must be transferred to pre-cooled equipment. The vapor pressure follows the equation $\log p = 7.689 - (1555/T)$, where p is in mm. Hg and T is in absolute temperature units.

The diluted halothane-tritium can be counted in a liquid scintillation counter at 22% efficiency and it can be assayed in concentrations of less than 0.1 mg. with suitable statistical accuracy. This material is being used to calibrate an infrared method of measuring halothane after it is extracted from blood.

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A Study of Aqueous Medicinal Lubricants*

By GERHARD LEVY† and T. W. SCHWARZ

The relation of viscosity and solid concentration to the lubricant effect of aqueous solutions of methylcellulose was investigated. Lubricity increased directly with solid concentration, while the high viscosity at greater methylcellulose concentrations prevented any further lubricity increase. Glycerin, polyethylene glycol 400, and propylene glycol are effective lubricants over a wide concentration range under the conditions tested.

THE FORMULATION of aqueous jellies and liquids designed to reduce the friction as well as the resulting pain and trauma encountered when inserting various devices into anatomical passages involves a consideration of the qualitative and quantitative effect of potential constituents on the lubricating property of the final product. In addition, the relationship between the rheologic characteristics of the product and its lubricating property must be established.

We have recently described a method for the measurement of the lubricating property of medicinal products by means of a specially designed instrument, the lubrimeter (1). The present report deals with a study of aqueous medicinal lubricants and the effect of viscosity, concentration of hydrocolloid, and addition of a polyol on lubricating property.

Friction, the resistance to motion when one solid body slides over another, is due to the junctions formed at the regions of real contact between the two bodies (2). Such areas of contact are very small relative to the actual surface area and involve only the tips of the asperities that are found on even the "smoothest" of surfaces. Ordinarily, the real area of contact is determined primarily by the load and the yield pressure of the materials and is not dependent on surface area. In the case of elastic or viscoelastic materials such as polyethylene or epithelium, the area of contact is no longer directly proportional to the load and as a result, the coefficient of friction increases as the load is reduced (2). For this reason, it is important that medicinal lubricants be tested under much lower loads than those utilized in evaluating engine lubricants.

A basic law of friction states that it is proportional to the load between surfaces. This law, however, holds only for dry friction, where the opposing surfaces are free of any film (3). It does not directly apply to skin and mucous membranes, another reason why the load must be kept low enough to be within the range commonly encountered in clinical practice.

For the purpose of this investigation of the relationship between viscosity and lubricant effect a series of solutions of two grades of methylcellulose, one a high molecular weight polymer and the other a low molecular weight polymer, were prepared and their lubricant effect evaluated. The two series of solutions may be expected to exhibit one basic type of flow characteristics. The individual solutions differ primarily in their viscosity and solid content. It was also our purpose to determine the lubricating effect of polyols, such as propylene glycol and glycerin, since these substances are common constituents of lubricating jellies, liquids, and lotions. The lubricating effects of the pure polyols, of their aqueous solutions, and of methycellulose solutions containing a given concentration of a polyol were established.

EXPERIMENTAL

Apparatus.—The "Lubrimeter" consists of a polished brass roller which is rotated by a synchromotor at various selected constant speeds. On this roller rests a drum which is covered with ribbed polyethylene sheeting. The drum is free to rotate around its own axis. The roller is partly immersed in a pan that serves as a reservoir for the lubricant. As the roller rotates, the drum turns in the opposite direction as a result of friction. The torque exerted on the drum is a measure of the frictional force and is determined by means of selected springs calibrated in grams. A detailed description of the instrument can be found in our previous report (1).

Procedure.—The procedure is identical to the one previously described (1). The reported friction values were determined at 25° ± 2.5° and were average values obtained from two series of six determinations each. The difference between the average values of the two series was less than 3%.

Materials.—The solutions were prepared from Methocel 90 HG 4000 and Methocel 90 HG 400 (hydroxypropyl methycellulose)[1] by adding the proper amount to distilled water previously heated to about 80°. The water contained 0.15% methylparaben and 0.025% propylparaben as preservatives. The solutions were then placed in a refrigerator for twenty-four hours.

Glycerin, propylene glycol, and polyethylene glycol 400 were of U. S. P. grade.

[1] Dow Chemical Co., Midland, Mich.

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† Present address: University of Buffalo, School of Pharmacy, Buffalo 14, N. Y.

Viscosity Determinations.—Viscosity measurements were made with the Brookfield viscometer, model LVF, at 25°, 60 r. p. m., after ten minutes of spindle rotation. The Brookfield viscosity units shown in Fig. 1 would, in the case of Newtonian liquids, represent centipoise.

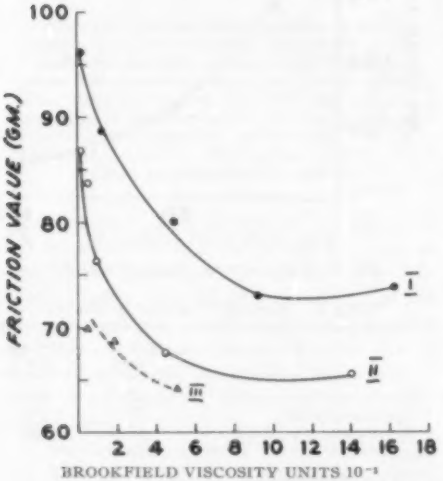

Fig. 1.—The relation of viscosity to lubricity. ●——● Methocel 90 HG 4000; ○——○ Methocel 90 HG 400; △- - -△ Methocel 90 HG 4000 with propylene glycol 20% v/v.

RESULTS AND DISCUSSION

The relationship between the viscosity and lubricant effect of methylcellulose solutions is shown in Fig. 1. Curve I represents solutions of Methocel 90 HG 4000, while curve II represents solutions of the lower molecular weight polymer, Methocel 90 HG 400. The rapid initial increase in lubricity, indicated in Fig. 1 by a decline of friction value, levels off at higher viscosities. In fact, curve I indicates a range of maximum lubricant effect and a slight decrease in lubricity at viscosities above the optimum range. It must be recognized that quantitatively this optimum range holds true only under the conditions of the test, and may vary with load and velocity of the moving surface.

The significance of viscosity in lubrication has been studied extensively in the engineering sciences. In instances of hydrodynamic ("full fluid") lubrication, the moving surfaces are completely separated by a continuous film of lubricant. Since it is generally accepted that there is no slip of the liquid at the solid surfaces, the resistance to motion (the resisting torque in the lubrimeter) is proportional to the coefficient of viscosity (4). If the lubricant exhibits Newtonian flow, the resistance to motion is also proportional to the velocity at which one solid surface moves relative to the opposing surface. These relationships are essentially based on Newton's law of viscous flow. Thus, resistance to motion is also inversely proportional to the clearance between opposing surfaces. These factors along with dimensional considerations constitute Petroff's equation which deals with the resisting torque encountered in lubricated bearings (5). This equation has been shown to provide reasonably accurate estimates of actual friction.

On the basis of the foregoing discussion, it appears that the lower the viscosity of the lubricant, the lower the resistance to motion. This is true only up to a certain limit. As the viscosity of the lubricant decreases, its "load-carrying" capacity also diminishes, and the distance between the opposing surfaces is reduced. If this distance becomes less than the height of the surface irregularities, direct contact between the opposing surfaces may be established and the coefficient of friction becomes much larger. The low "load-carrying" capacity at low viscosities and the resistance to motion encountered at high viscosities account for a range of optimum viscosity for lubrication under specified conditions.

The correlation of lubricity with the viscosity of non-Newtonian liquids, such as solutions of methylcellulose, is much more complex than the straightforward relation of viscosity of Newtonian liquids to their lubricant effect. The rate of shear, to which the solutions are subjected when they exist as a lubricant film between opposing surfaces, will vary extensively throughout the film because the clearance between the opposing surfaces varies widely and changes constantly. The viscosity of methylcellulose solutions changes accordingly, since the apparent viscosity coefficient of non-Newtonians is dependent on the rate of shear. Our choice of shear rate for the determination of viscosity had to be arbitrary and, therefore, the correlation of non-Newtonian viscosity with lubricant effect can, at best, be only descriptive.

A comparison of curves I and II in Fig. I shows that, at equal viscosities, the lubricant effect of the two grades of methylcellulose differs markedly. The solid content of the Methocel 400 solutions is much greater than that of the Methocel 4000 solutions of equal viscosity by reason of the lower degree of polymerization of the former. Figure 2 shows a plot of friction values against per cent solid content. Significantly, the lubricant effect increased (i. e., friction values decreased) in linear fashion with increasing solid content. Moreover, there was relatively little difference between the two grades of methylcellulose; they could almost be plotted on the same curve. This linear relationship holds true up to a certain point, at which a sudden cut-off or levelling-off occurs. By comparison with Fig. 1, it can be seen that this cut-off occurs when the viscosity becomes sufficiently high to interfere with lubrication.

The data appear to indicate that the lubricant quality of methylcellulose solutions is primarily a function of their solid content. High viscosity as a result of increasing solid concentrations limits further lowering of the friction value. Beyond a certain viscosity, lubricity does not increase and apparently even decreases, as curve I, Fig. 1 indicates.

These observations may be explained in the light of the experiences gained by lubrication engineers. At low sliding speeds which are representative of clinical conditions, the lubricant film may

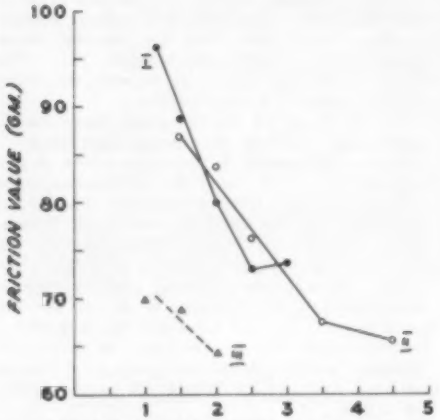

Fig. 2.—The relation of solid concentration to lubricity. ●——● Methocel 90 HG 4000; ○——○ Methocel 90 HG 400; △- - - -△ Methocel 90 HG 4000 with propylene glycol 20% v/v.

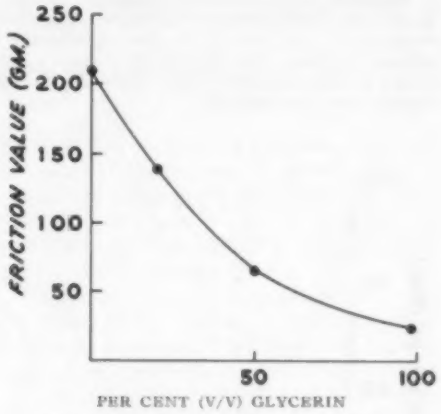

Fig. 3.—The lubricity of glycerin U.S.P.

break down and a partial transition of hydrodynamic lubrication to boundary lubrication may take place. The latter is a condition where the sliding surfaces are separated by very thin lubricant films which at times may be only a few molecules thick. In this case, viscosity is less important as a determining factor for lubricity than the chemical constitution of the lubricant.

Effective boundary lubrication requires the presence of a layer of lubricant molecules on the sliding surfaces. Higher concentrations of methylcellulose in the lubricant solution should provide a more compact surface layer of the hydrocolloid. Since equal concentrations by weight of the polymer, regardless of the degree of polymerization, will provide about the same number of monomer units per volume of solution, it may be expected that equal concentrations by weight of methylcellulose, regardless of molecular weight, will exhibit a similar lubricant effect. This was found to be the case in our investigation up to the point where high viscosity interferes with lubrication.

Figure 3 represents the relation of concentration to the lubricant property of aqueous glycerin solutions. The graph shows that glycerin is an effective lubricant under the conditions tested. The lubricant properties increase with concentration, but are significant even in aqueous solutions containing only 20% glycerin. Significantly, the major increase in lubricity occurs in the lower concentration region when the viscosity changes are minor. At higher glycerin concentrations (above 50%), the changes in lubricity are not as great, while the viscosity increases profoundly.

An examination of two other polyols commonly used as components of medicinal lubricants, propylene glycol and polyethylene glycol 400, shows that their lubricant effect does not differ significantly from that of glycerin under the conditions tested. The results are tabulated in Table I.

Finally, it is of interest to determine the effect of a polyol when added to a solution of a hydrocolloid such as methylcellulose. This is shown by curve III in both Figs. 1 and 2. In both instances, regardless of whether friction value is plotted against viscosity or solid concentration, it can be seen that the lubricant effect increases significantly upon the addition of propylene glycol.

TABLE I.—FRICTION VALUES OF POLYOLS

	U. S. P. Grade, Gm.	20% Aq. Soln., Gm.
Glycerin	22.7	138
Propylene glycol	22.0	141
Polyethylene glycol 400	17.9	130
Water[a]	210.0	...

[a] Added for comparison.

SUMMARY AND CONCLUSIONS

1. The effect of viscosity and solid concentration on the lubricity of aqueous methylcellulose solutions was investigated with the lubrimeter. The lubricating effect of selected polyols was also investigated.

2. The results indicate that the lubricating effect of methylcellulose solutions increases directly with solid concentration, while the concomitantly increasing viscosity becomes the limiting factor.

3. Glycerin, propylene glycol, and polyethylene glycol 400 are effective lubricants. Propylene glycol enhanced the lubricity of methylcellulose solutions.

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Ultraviolet Photometric Assay of Thiopental and Pentobarbital in Blood and Plasma*

By STEPHEN I. OROSZLAN and GERTRUDE D. MAENGWYN-DAVIES

A procedure for the determination of sodium pentobarbital and sodium thiopental under rigidly standardized conditions of extraction, using Allen's absorption factor, is described. This method makes possible the reliable quantitative determination of 1 mcg./ml. or smaller concentration differences. In an appendix Allen's absorption factor for sodium pentobarbital is derived.

A CORRECTION FACTOR derived by Allen (1) is used routinely in the quantitative spectrophotometric estimation of steroids. This absorption factor, derived from a reading at the peak of absorption and at two wavelengths equidistant below and above the peak, can be employed when the background absorption is linear or nearly linear within the three wavelengths used.[1]

It was already reported by Broughton (2) that a linear absorption spectrum is obtained in blood extracts of approximately pH 13.5 (in 0.45 *N* NaOH) in the U. V. region where the barbiturates exhibit their characteristic absorption maxima at that pH. We therefore investigated whether or not a more sensitive method for the determination of pentobarbital and thiopental might be devised, under rigidly standardized conditions, employing Allen's correction factor.

EXPERIMENTAL

Reagents

1. NaOH stock solution, 50% (w/v). (*a*) NaOH, 0.45 *N*, made fresh daily from NaOH stock solution and (*b*) NaOH, 0.2 *N*, made fresh daily from NaOH stock solution.
2. Chloroform, reagent grade (certified, Fisher Scientific Co.), washed daily with 1 *N* NaOH and distilled water, according to Goldbaum (3).
3. NaCl, reagent grade (Merck and Co., Inc.), 0.85% solution.
4. Sodium pentobarbital (Nembutal Sodium, Abbott Laboratories) stock solution (0.4 mg./ml.) in 0.45 *N* NaOH, freshly prepared daily and kept refrigerated.
5. Sodium thiopental (Pentothal Sodium, Abbott Laboratories) stock solution (0.1 mg./ml.) in 0.2 *N* NaOH, freshly prepared daily and used immediately after preparation.

All solutions were made with glass distilled water.

Procedure

Preparation of Standard Curve for Sodium Pentobarbital.—Appropriate dilutions of stock solution No. 4 were made in 0.45 *N* NaOH and read in the Beckman DU spectrophotometer in silica cells (1-cm. light path; slit width, 0.22; temperature, 22°) at three wavelengths, 245, 255 (absorption maximum), and 265 mμ. NaOH, 0.45 *N*, served as reference solution. Calculations, using Allen's (1) factor, were made as follows:

$$F = A_{255} - \frac{A_{245} + A_{265}}{2}$$

Sodium pentobarbital was determined in the range of 5 to 25 mcg./ml. The standard curve, shown in Fig. 1, was obtained by plotting F on the ordinate against mcg./ml. of the drug on the abscissa. Since a straight line is obtained for this plot, it obeys Beer-Lambert's law.

Preparation of Standard Curve for Sodium Thiopental.—This standard curve, also shown in Fig. 1, was made by appropriate dilutions of the stock solution No. 5 in 0.2 *N* NaOH, and read against 0.2 *N* NaOH as reference solution in the Beckman DU spectrophotometer under the same conditions as described above. The maximum absorption for sodium thiopental was obtained at 305 mμ, and therefore, two more readings were taken at 295 and 315 mμ. The standard curve for this drug, obtained in the range of 1 to 10 mcg./ml., was calculated as follows:

$$F = A_{305} - \frac{A_{295} + A_{315}}{2}$$

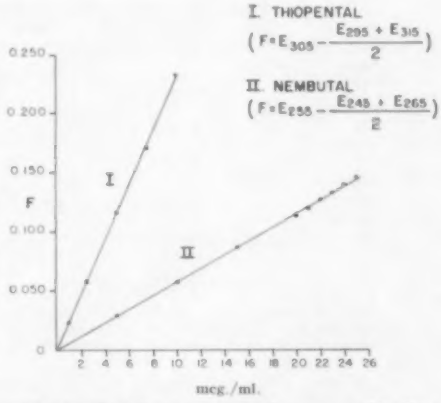

Fig. 1.—Barbiturate standard curves. Abscissa: mcg./ml. of the barbiturates. Ordinate: $F_{thiopental}$ (I) at pH 13.2 and $F_{nembutal}$ (II) at pH 13.45.

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[1] See Appendix.

As can be seen from Fig. 1, in the range studied this compound also obeyed Beer-Lambert's law, when F was plotted against the drug concentration (mcg./ml.).

Extraction from Whole Blood or Plasma.—Dated plasma from the blood bank or whole oxalated dog blood was used for this purpose. Appropriate concentrations of sodium pentobarbital were dissolved in 0.85% NaCl, so that upon the addition of 0.1 ml. of the barbiturate to exactly 4.0 ml. of plasma or whole blood, a final barbiturate concentration of 50, 40, 30, and 20 mcg./ml. was obtained (samples). A reference solution (blank) was prepared by adding to a 4.0-ml. aliquot of plasma or whole blood 0.1 ml. of 0.85% NaCl. Samples and blank in triplicates were extracted with 40 ml. of chloroform in glass-stoppered bottles (125 ml.) on a variable speed shaker (Arthur H. Thomas Co.), set always at an identical slow speed setting. The bottles were shaken for exactly thirty minutes at room temperature. The speed and timing was chosen so that nearly complete extraction was obtained without the formation of emulsions. Most of the chloroform layer was siphoned off with a glass serum lifter and filtered through Whatman No. 1 filter paper. The initial 2 ml. of the filtrate was discarded, the rest collected, and exactly 20.0 ml. of the filtrate re-extracted with 4.0 ml. of the 0.45 N NaOH (pentobarbital) or 0.2 N NaOH (thiopental) in glass-stoppered, 25-ml. cylinders by vigorous shaking by hand for one minute. After separation of the two layers, the barbiturate was determined in the clear upper layer as described for the standard curves and read against 0.45 N or 0.2 N NaOH, respectively. The absorption of the blanks was determined to insure that the essential condition for the application of the Allen factor (1) (linearity) was met. In Table I, a recovery experiment of triplicate extractions for whole blood is summarized.

Comparison of the Sensitivity of This Method with Known Methods.—Sodium pentobarbital standard curves were prepared in triplicate, according to our method, Broughton's (2) method, and Goldbaum's (3) method. The results obtained are summarized in Table II.

The readings given are the arithmetic means of the triplicate determinations. The overall means and standard deviations of the means for 1 mcg./ml. of sodium pentobarbital were calculated using all results obtained, and are shown in the last two lines of Table II.

TABLE I.—RECOVERY OF PENTOBARBITAL FROM BLOOD

Added, mcg./ml.	Recovered, mcg./ml.	Recovery, %
50	49.4	98.80
40	39.1	97.75
30	29.4	98.00
20	20.4	102.00

DISCUSSION

It is obvious from our results that Allen's (1) factor (F) can be applied to the quantitative ultraviolet determination of barbiturates. An inspection of Table II clearly indicates that with the aid of F, a far more reliable and sensitive method for quantitative barbiturate analysis is made possible. The error involved for the determination of 1 mcg./ml. of pentobarbital, when trying to differentiate small concentration differences, is: 10.5% by Goldbaum's method (3), 28.7% by Broughton's (2) method, 12.7% by direct alkaline U. V., and 5.4% by our method. Also, our recovery data show an average of 99.14% by our method, and 95% by Goldbaum's (3) method, as determined by us under carefully standardized conditions of extraction procedure. We therefore believe that our method, using a rigidly standardized extraction procedure and employing Allen's (1) factor for the readings, yields a considerably more sensitive and reliable barbiturate assay. In our case we read the same final solution at three different wavelengths at the same pH. By the other methods, further dilutions and treatment of the barbiturate solutions to

TABLE II.—COMPARISON OF PENTOBARBITAL STANDARD CURVES BY DIFFERENT U. V.-SPECTROPHOTOMETRIC METHODS

Pentobarbital Na, mcg./ml.	Our Method, F	A at Absorption Maximum[a]	Goldbaum's Method (3), $A_{diff.}$[b]	Broughton's Method (2), $A_{diff.}$[c]
20	0.113	0.537	...	0.386
20.4	...	...	0.435	...
21	0.120	0.581	...	0.409
21.6	...	...	0.461	...
22	0.127	0.607	...	0.425
22.8	...	...	0.479	...
23	0.133	0.631	...	0.427
24	0.139	0.658	0.501	0.452
25	0.147	0.697	...	0.461
Mean increase for 1 mcg./ml. concentration increment	$F \times 10^3$ = 6.80	A_{255} mμ $\times 10^3$ = 32.00	$A_{diff.} \times 10^3$ = 18.33	$A_{diff.} \times 10^3$ = 15.00
±S. E. of mean $\times 10^3$ =	±0.37	±3.98	±1.92	±4.31

[a] A = Absorbance at 255 mμ at pH 13.5 (in 0.45 N NaOH).
[b] $A_{diff.}$ (Absorbance difference) = (A at 260 mμ at pH 13.5) − (A at 260 mμ at pH 10.5).
[c] $A_{diff.}$ = (Absorbance at 260 mμ at pH 13.5) − (Absorbance at 260 mμ at pH 10.0).

obtain a lower pH are necessary, which might account for the increase in error.

No doubt, all barbiturates which can be determined by other methods can also be quantitated employing Allen's (1) factor in our modification of Goldbaum's (3) method. This more sensitive method may not be necessary for clinical barbiturate determinations, where such small concentration differences could be overlooked, but in the pharmaceutical assay of some preparations containing barbiturates and in the pharmacological study of barbiturate metabolism this modification might prove to be useful.

APPENDIX

The arithmetic derivations below support the conclusions indicated by the equations which define Allen's factor (1). This factor can be used to correct for any quantitative absorption of mixtures where one component shows a linear absorption in the region where the other component has a peak, i. e., barbiturate determination in blood, the resolution of mixtures of some barbiturates, etc. The mathematical treatment is a quantitative statement of the intuitively obvious. $(A)^B$ = absorbance of blood extract; $(A)^N$ = absorbance of pure pentobarbital-Na; $(A)^{BN} = (A)^N + (A)^B$ = absorbance of pentobarbital-Na extracted from blood; F = correction factor,[1] with subscripts B, N, BN, having the same meaning as the above superscripts.

With a linear background absorption (Line 1) and equidistance of a and c from b, we show two identical, rectangular triangles with the angle α, therefore

$$b = a + h = c - h \qquad \text{(Eq. 1)}$$

and

$$2b = (a + h) + (c - h) \qquad \text{(Eq. 2a)}$$

or

$$b = (a + c)/2 \qquad \text{(Eq. 2b)}$$

therefore

$$F_B = (A)^B_{255} - \frac{(A)^B_{245} + (A)^B_{265}}{2} = b - \frac{a + c}{2} = 0 \qquad \text{(Eq. 3)}$$

$$F_N = (A)^N_{255} - \frac{(A)^N_{245} + (A)^N_{265}}{2} \qquad \text{(Eq. 4)}$$

and by substitution

$$F_N = (2b + e + g) - \frac{(a + d) + (c + f)}{2} \qquad \text{(Eq. 5)}$$

[1] On the diagram (Fig. 2) the designation of the ordinates (A-values) are as follows: $(A)^B_{245} = a$; $(A)^B_{255} = c$; $(A)^B_{265} = b$; $(A)^N_{245} = a + d$; $(A)^N_{255} = c + f$; $(A)^N_{265} = 2b + e + g$; (lines 4 and 5 cut off a distance from this ordinate which is equal to b).

Since the absorbances of pure nembutal and the blood extract impurity are additive, it follows that: $(A)^{BN}_{245} = 2a + d$; $(A)^{BN}_{255} = 2c + f$; $(A)^{BN}_{265} = 3b + e + g$.

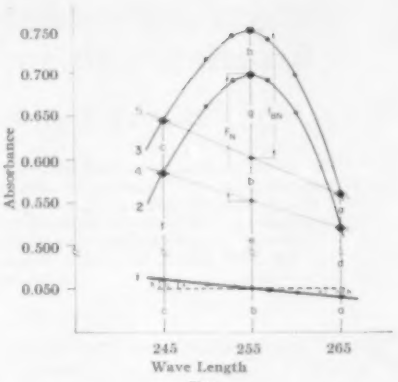

Fig. 2.—Diagram for derivation of Allen's correction factor. Line 1, background absorption of blood extract in 0.45 N NaOH; curve 2, absorption of pure pentobarbital-Na (25 mcg./ml.) in 0.45 N NaOH; and curve 3, absorption of pentobarbital-Na (25 mcg./ml.) extracted from blood in 0.45 N NaOH.

In accordance with the derivation of Eq. 2b

$$\frac{(a + d) + (c + f)}{2} = (e + b) \qquad \text{(Eq. 6)}$$

therefore

$$F_N = (2b + e + g) - (e + b) \qquad \text{(Eq. 7a)}$$

or

$$F_N = b + g \qquad \text{(Eq. 7b)}$$

$$F_{NB} = \frac{(A)^{BN}_{255} - (A)^{BN}_{265} + (A)^{BN}_{245}}{2} \qquad \text{(Eq. 8)}$$

and by substitution

$$F_{BN} = (3b + e + g) - \frac{(2a + d) + (2c + f)}{2} \qquad \text{(Eq. 9)}$$

In accordance with the derivation of Eq. 2b

$$\frac{(2a + d) + (2c + f)}{2} = (2b + e) \qquad \text{(Eq. 10)}$$

therefore

$$F_{BN} = (3b + e + g) - (2b + e) \qquad \text{(Eq. 11a)}$$

or

$$F_{BN} = b + g \qquad \text{(Eq. 11b)}$$

From Eq. 7b and 11b

$$F_N = F_{BN} = b + g \qquad \text{(Eq. 12)}$$

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Effects of Psychopharmacologic Agents on Experimentally-Induced Seizures in Mice*

By GREGORY B. FINK† and EWART A. SWINYARD

Of seven psychopharmacologic drugs screened by five assay procedures, only meprobamate and phenaglycodol exhibited significant anticonvulsant activity. Chlorpromazine, promazine, reserpine, and hydroxyzine lowered the threshold for minimal seizures variously induced, whereas trifluprom azine had no effect on seizure threshold. The data obtained correlate well with available clinical data and indicate the value of threshold seizure studies in animals for detecting agents which may cause convulsions in man.

DESPITE THE CONSIDERABLE attention given to both the anticonvulsant and the convulsant properties of psychopharmacologic agents, there is a lack of agreement as to the ability of these agents to alter the seizure threshold in laboratory animals and man. In laboratory animals, chlorpromazine has been reported both to lower seizure threshold (1, 2) and to exhibit anticonvulsant properties (3). In man, reserpine has been reported both to increase (4) and to decrease (5, 6) the incidence of convulsive seizures in mentally ill epileptics. Furthermore, published experimental data have been difficult to compare, because of differences in the techniques employed in various laboratories, and because the drugs under scrutiny were usually tested by only one or two assay procedures. Therefore, it was thought important to make a critical study of the effects of selected psychopharmacologic agents on experimentally-induced seizures by a battery of well-standardized methods. For comparative purposes, diphenylhydantoin and phenobarbital were also included as classical prototype anticonvulsants.

METHODS

Male albino mice (Carworth Farms, CF No. 1 strain) weighing 22 to 36 Gm. were used as experimental animals. They were maintained on Rockland mouse diet and allowed free access to food and water except for the short time they were removed from their cages for testing. The following agents were studied: chlorpromazine hydrochloride (Thorazine), promazine hydrochloride (Sparine), trifluprom azine hydrochloride (Vesprin), reserpine, hydroxyzine hydrochloride (Atarax), meprobamate (Equanil, Miltown), phenaglycodol (Ultran), phenobarbital sodium, and diphenylhydantoin sodium. All drugs were given orally in aqueous solution, except that meprobamate and phenaglycodol were given as suspensions in 6% acacia solution.

Anticonvulsant Assay Methods.—Anticonvulsant potencies (ED_{50}s) were determined by a battery of four electrical tests and one chemical test. The tests based on electrically-induced convulsions measured the ability of a drug to prevent the hindleg tonic-extensor component of maximal electroshock seizures evoked by supramaximal current (MES test; 50 ma. alternating current, 0.2-second stimulus duration), to elevate the threshold for minimal electroshock seizures (alternating current, 0.2-second stimulus duration) in normal mice (a. c. EST test) and in hyponatremic (low-threshold) mice (HET test), and to elevate the threshold for low-frequency electroshock seizures (l. f. EST) induced in mice by unidirectional current delivered at an intensity twice threshold (0.2-millisecond duration, 3-second stimulus duration, six pulses per second). The test based on chemically-induced convulsions measured the ability of a drug to afford complete protection against seizures evoked by the subcutaneous injection of pentylenetetrazol (Metrazol; 85 mg./Kg.; s. c. Met. test). Except that a Grass stimulator (model S4B) was used for the l. f. EST test, the details of the various procedures, the end points employed in mice, and the characteristics of the electroshock apparatus have been described elsewhere (7–9). In addition, the mean neurotoxic dose (TD_{50}) and the mean lethal dose (LD_{50}) were determined for each drug. The end point for minimal neurotoxicity was muscular incoordination, based on inability of the animal to remain for one minute on a horizontal rod rotating at 5 revolutions per minute. Each drug was tested at the time of its peak activity as measured by the neurotoxicity test. For the determination of the ED_{50}, TD_{50}, or LD_{50}, groups of six or more mice were given various doses of drug until at least three points were established in the range between 0 and 100% seizure protection, minimal neurotoxicity, or mortality. The results obtained were then plotted on logarithmic probability paper and a regression line was fitted to the plotted points by eye. From this plot of the data the respective ED_{50}, TD_{50}, or LD_{50} was determined and the protective indexes (PI = TD_{50}/ED_{50}) were computed, 95% fiducial limits were calculated by the method of Litchfield and Wilcoxon (10).

Convulsant Assay Methods.—Drugs which lacked anticonvulsant activity were examined for convulsant properties by the three electroshock threshold procedures (l. f. EST, a. c. EST, and HET) mentioned above, except that the experimental design

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† Predoctoral trainee under National Institutes of Health Pharmacology Training Grant (2G-153).

TABLE I.—TOXICITY AND ANTICONVULSANT ACTIVITY OF SOME PSYCHOPHARMACOLOGIC DRUGS

Drug	Time of test, min.	LD_{50} mg./Kg.	TD_{50} mg./Kg.	ED_{50}, mg./Kg. MES	a. c. EST	HET	l. f. EST	s. c. Met.
Diphenylhydantoin	180	490 (295–808)[a]	84 (74.3–94.8)	14.4 (12.0–17.3)	>200	37 (24.6–55.3)	25 (16.7–37.5)	>200
Phenobarbital	180	200 (167–240)	70 (58.3–84.0)	30 (23.6–38.1)	33 (21.3–51.1)	26 (17.3–39.0)	20 (13.3–30.0)	18.5 (12.7–26.8)
Meprobamate	30	1,350 (1,205–1,512)	228 (196–262)	187 (147–237)	190 (152–238)	190 (138–260)	123 (89.8–168)	89 (73.1–119)
Phenaglycodol	60	760 (678–852)	170 (119–241)	135 (120–151)	100 (62.9–159)	80 (57.6–111)	73 (59.3–89.7)	59 (48.0–72.5)
Chlorpromazine	90	580 (460–730)	15.7 (10.6–23.2)	261 (209–326)	>200	>200	>80	>300
Promazine	60	485 (323–727)	23.5 (15.0–36.9)	175 (152–201)	>200	>150	>100	>150
Trifluprom azine	90	330 (206–512)	7.8 (5.3–11.5)	>300	>200	>150	>50	>200
Reserpine	240	390 (265–573)	16.5 (11.6–23.4)	>300	>200	>200	>33	>200
Hydroxyzine	30	515 (464–571)	490 (434–553)	>350	>350	>350	>350	>350

[a] Values in parentheses are 95% fiducial limits.

was modified to measure seizure threshold quantitatively. In addition, pentylenetrazol seizure threshold (i. v. Met.) was measured by the technique of Orloff, *et al.* (11), as modified by McQuarrie and Fingl (12). For each electroshock procedure, 60 or more mice were randomly divided into two equal groups; for the chemoshock procedure, 20 or more mice similarly were randomly divided into two equal groups. One group of mice was given the drug to be tested and the other (control) group was given the requisite volume of vehicle. At the previously determined time of peak drug effect, seizure threshold was determined in both groups by technique previously described (12, 13, 14). Results are presented as threshold ratios (threshold of drug-treated group/threshold of control group). Threshold ratios were determined at dose levels equivalent to fractions and multiples of the TD_{50}s of the several drugs.

RESULTS

The time of peak effect, neurotoxicity, and anticonvulsant activity of the agents tested are summarized in Table I. The time of peak effect of the several agents varied from thirty minutes for meprobamate and hydroxyzine to as long as two hundred and forty minutes for reserpine. Phenobarbital had the lowest LD_{50} (forty-eight-hour observation period) and the two substituted diols the highest. The LD_{50}s of the other six drugs ranked intermediate to those for phenobarbital and the substituted diols and in most instances were not significantly different from each other. The TD_{50}s for the nine drugs varied from 7.8 mg./Kg. for trifluprom azine, the most neurotoxic, to 490 mg./Kg. for hydroxyzine, the least neurotoxic. The remaining seven compounds can be divided into the following groups ranked in order of increasing TD_{50}s: (*a*) chlorpromazine, promazine, and reserpine, (*b*) diphenylhydantoin and phenobarbital, and (*c*) meprobamate and phenaglycodol. There is no significant difference in the TD_{50}s of the drugs within each group.

With regard to anticonvulsant activity, only phenobarbital and the two substituted diols were effective by all five tests. All three drugs were the most potent by the s. c. Met. test; the ED_{50}s for phenobarbital, meprobamate, and phenaglycodol by this test were 18.5, 89, and 59 mg./Kg., respectively. Diphenylhydantoin exhibited the lowest ED_{50} (14.4 mg./Kg.) in the MES test, was effective in nontoxic doses by the HET and l. f. EST tests, but was inactive by the a. c. EST and s. c. Met. tests. The remaining five drugs, chlorpromazine, promazine, trifluprom azine, reserpine, and hydroxyzine, were ineffective anticonvulsants by all five tests, except that highly toxic doses of chlorpromazine and promazine (261 and 175 mg./Kg., respectively) abolished the tonic-extensor component of the MES pattern. Reserpine, in fact, increased the severity of experimentally-induced seizures, and many reserpine-treated mice died following the convulsions.

The drugs which were ineffective by the five anticonvulsant tests were studied for ability to lower the threshold for electrically and chemically-induced seizures. Threshold ratios (threshold of drug group/threshold of control group), as shown in Table II, were determined at fractions and multiples of the TD_{50} for the phenothiazines and reserpine, and at fractions and multiples of the LD_5 (350 mg./Kg.) for hydroxyzine. Chlorpromazine significantly lowered l. f. EST and a. c. EST at $^1/_2$ the TD_{50} (7.8 mg./Kg.) and the TD_{50} (15.7 mg./Kg.), but it did not alter the l. f. EST at twice the TD_{50} (31.4 mg./Kg.). Promazine significantly lowered both l. f. EST and a. c. EST at all dose levels employed, except for the a. c. EST at $^1/_2$ the TD_{50} (11.7 mg./Kg.) the decrease was not significant; even at 4 times the TD_{50} (94 mg./Kg.) the decrease in l. f. EST was significant. The other phenothiazine, trifluprom azine, had no significant effect on electroshock seizure thresholds. Reserpine also lowered electroshock

TABLE II.—EFFECTS OF SOME PSYCHOPHARMACOLOGIC DRUGS ON THE THRESHOLD FOR ELECTRICALLY- AND CHEMICALLY-INDUCED SEIZURES IN MICE

Drug	Dose mg./Kg.	Threshold Ratios[a]			
		i. f. EST	a. c. EST	HET	i. v. Met.
Chlorpromazine	7.8	0.86[b]	0.90[b]	...	1.04
	15.7	0.83[b]	0.84[b]	0.90	0.90
	31.4	0.99	...	...	0.96
Promazine	11.7	0.85[b]	0.91	...	0.80[b]
	23.5	0.79[b]	0.89[b]	0.93	0.86[b]
	47.0	0.84[b]	...	...	0.90
	94.0	0.81[b]	...	...	...
Trifluprom azine	3.9	1.04	1.00	...	...
	7.8	0.93	1.00	0.94	1.02
	15.6	0.99	...	...	1.05
Reserpine	4.1	0.89	0.86[b]	...	...
	8.2	0.71[b]	0.79[b]	0.93	0.91
	16.5	...	0.88[b]	...	0.76[b]
	33.0	...	0.80[b]	...	...
Hydroxyzine	87.5	0.92	1.01	...	0.79[b]
	175.0	0.96	0.93	1.04	0.86[b]
	350.0[c]	0.98	0.98	...	...

[a] Threshold of drug-treated group/threshold of control group. [b] Significant decrease ($P < 0.05$). [c] LD_5.

TABLE III.—PROTECTIVE INDEXES OF SOME ANTIEPILEPTIC AND PSYCHOPHARMACOLOGIC DRUGS BY FIVE ASSAY METHODS IN MICE

Drug	Protective Indexes[a]				
	MES	a. c. EST	HET	i. f. EST	s. c. Met.
Diphenylhydantoin	5.8 (4.7–7.3)[b]	...[c]	2.3 (1.5–3.5)	3.4 (2.2–5.1)	...[c]
Phenobarbital	2.3 (1.7–3.2)	2.1 (1.3–3.4)	2.7 (1.7–4.2)	3.5 (2.3–5.4)	3.8 (2.5–5.7)
Meprobamate	1.2 (0.9–1.6)	1.2 (0.9–1.7)	1.2 (0.9–1.7)	1.9 (1.3–2.6)	2.6 (1.8–3.6)
Phenaglycodol	1.3 (0.9–1.8)	1.7 (0.9–3.1)	2.1 (1.3–3.5)	2.3 (1.6–3.5)	2.9 (1.9–4.3)

[a] PI = TD_{50}/ED_{50}. [b] Values in parentheses are 95% fiducial limits. [c] Inactive by this test.

seizure thresholds at all dose levels, except that at a dose of 1/4 the TD_{50} (4.1 mg./Kg.) the decrease in l. f. EST was not significant. In doses up to the LD_5 (350 mg./Kg.), hydroxyzine had no significant effect on the electroshock seizure thresholds. None of the drugs had any significant effect on the HET at the dose levels employed. Promazine was the only phenothiazine to have a significant effect on the chemoshock seizure threshold. This drug significantly lowered i. v. Metrazol threshold at 11.7 and 23.5 mg./Kg., whereas chlorpromazine and trifluprom azine had no effect, even in doses twice the TD_{50} (31.4 and 15.6 mg./Kg., respectively). Reserpine significantly reduced the chemoshock seizure threshold at the TD_{50} (16.5 mg./Kg.) but not at 1/3 the TD_{50}. Many reserpine-treated mice exhibited a maximal seizure immediately following the clonic seizure and expired, this rarely occurred in mice given other drugs. Hydroxyzine also lowered the i. v. Metrazol threshold in nontoxic doses of 87.5 and 175 mg./Kg.

DISCUSSION

The data presented show a wide latitude in the LD_{50}s and TD_{50}s of the nine drugs studied. The LD_{50}s indicate that phenobarbital is the most toxic and meprobamate the least toxic, when death is taken as the end point. On the other hand, the TD_{50}s indicate that trifluprom azine is the most toxic and hydroxyzine the least toxic, when minimal overt evidence of muscular incoordination is taken as the end point. Ratios obtained by dividing the LD_{50} by the TD_{50} of each drug indicate that the phenothiazines and reserpine have the highest ratios; diphenylhydantoin, phenobarbital, and substituted diols have intermediate ratios; and hydroxyzine has the lowest ratio of the nine drugs studied. For example, the ratio for trifluprom azine is 42.3; in marked contrast, hydroxyzine has a ratio of only 1.05. These observations emphasize that both the LD_{50} and the TD_{50} should be considered when estimating the toxicity of a new drug.

Only four drugs, diphenylhydantoin, phenobarbital, meprobamate, and phenaglycodol, exhibited anticonvulsant activity. Their relative anticonvulsant effectiveness, expressed as protective indexes (PI = TD_{50}/ED_{50}), are shown in Table III. Diphenylhydantoin has the highest protective index by the MES pattern test, but it is ineffective by two of the four threshold tests. Phenobarbital, meprobamate, and phenaglycodol are effective in nontoxic doses by all five tests; hence, they exhibit PIs greater than 1.0. Thus, in mice, the two substituted diols have margins of safety and profiles of anticonvulsant activity quite similar to those of phenobarbital. These experimental results are in agreement with clinical reports which indicate that meprobamate (15–17) and phenaglycodol (18) are of some clinical value in certain types of epilepsy.

The anticonvulsant data reported herein corroborate previous reports on the unique profile of action of diphenylhydantoin (7, 8, 19). The data also agree with our previous experiments which show that the two substituted diols resemble phenobarbital in that they modify both maximal audiogenic and maximal electroshock seizure patterns (20) and elevate the threshold for l. f. EST and i. v. Met. (13, 14, 21). Other workers have also commented on the barbiturate-like action of meprobamate (22). Several reports from our laboratories have emphasized that diphenylhydantoin is more effective in preventing seizure spread than in elevating seizure threshold, whereas phenobarbital both prevents seizure spread and elevates seizure threshold (19, 23). Since the substituted diols exhibit a profile of activity which resembles that of phenobarbital rather than diphenylhydantoin, their anticonvulsant activity would seem to be due to both mechanisms of action.

The results obtained by the convulsant assay methods agree with reported clinical observations that certain of the agents studied can induce seizures, and suggest that these tests may be of value for revealing seizure-evoking properties of new psychopharmacologic drugs. Chlorpromazine, promazine, and reserpine significantly lower both l. f. EST and a. c. EST, whereas promazine, reserpine, and hydroxyzine lower i. v. Met. threshold; all of these agents have been reported to cause convulsions in man, to increase the frequency of seizures in epileptic patients, or to increase fatalities in patients undergoing electroconvulsive therapy (24–34). On the other hand, trifluprom azine had no significant effect on either electroshock or chemoshock seizure thresholds; in so far as we are aware, this particular agent has not been reported to cause seizures in man.

SUMMARY

Seven psychopharmacologic and two antiepileptic agents were screened by a battery of five anticonvulsant assay procedures, and the drugs found ineffective were subjected to seizure threshold studies to determine whether they had potential convulsant properties. The mean neurotoxic dose and the mean lethal dose of each drug were determined. Meprobamate and phenaglycodol were effective in nontoxic doses by all five anticonvulsant tests and exhibited a profile of activity which resembled that of phenobarbital. Chlorpromazine lowered the threshold for alternating current and low-frequency electroshock seizure threshold (a. c. and l. f. EST); promazine lowered not only the a. c. and l. f. EST but also the intravenous pentylenetrazol threshold (i. v. Met.); trifluprom azine had no effect on these experimental seizure thresholds. Reserpine decreased all three types of seizure thresholds and facilitated convulsive activity. Hydroxyzine had no effect on electroshock seizure thresholds but lowered the i. v. Met. threshold. It was concluded that the several tests employed have only limited value for screening potential psychopharmacologic agents, but that they are definitely useful for disclosing their neuropharmacological properties and for detecting any propensity to induce seizures in man.

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The Facilitative Action of Reserpine on Metrazol Convulsions When Modified by Iproniazid*

By L. R. WEISS†, J. W. NELSON, and A. TYE

The effects of reserpine, iproniazid, and their combination on the convulsive threshold, facilitative action, and seizure pattern of metrazol-treated mice was investigated. The effect of reserpine on metrazol convulsions is a marked decrease in convulsive dose which is accompanied by a reduced seizure latency time in the convulsive pattern with an increasing facilitative action. Iproniazid was found to increase the convulsive dose and lengthen the latency interval. Prior treatment with iproniazid before reserpine reversed the depressive state produced by the latter agent to that of stimulation. This was accompanied by an increased anticonvulsant effect, longer latency interval, and absence of a facilitative action. Treatment with iproniazid after reserpine failed to elicit these effects. These results are compatible with the hypothesis that iproniazid and reserpine produce their effect through a biochemical change, the former by monoamine oxidase inhibition and the latter by release of norepinephrine and other amines from their storage sites in the central nervous system.

THE EFFECTS of reserpine on metrazol shock in mice were reported by Chen and Bohner (1). They found that pretreatment with reserpine modified the convulsive pattern by shortening the interval between the first sign of clonic movements and the final tonic extension of the hindlimbs in mice. This interval is termed the seizure latency time in the convulsive pattern. Another observation was the decrease in the convulsive threshold which is manifested by a reduction in the dose of metrazol required to produce a comparable response. This reduced shock threshold and decreased seizure latency period appears to be a characteristic of reserpine. The central depression seen after reserpine administration, therefore, possesses a convulsive component—a contradiction to the classical anticonvulsant action of many central depressants. Moreover, with a general depression of motor activity accompanied by sedation, a marked potentiating effect on the hypnotic action of barbiturates and other central depressants is commonly seen (2). The nature of this dual mechanism involving both hypnotic potentiation and convulsive phenomena is still not clear. The current belief is that various indole and catecholamines which are found in the biochemical and metabolic pathways within the central nervous system are influenced by the action of reserpine (3, 4). The varied actions of reserpine are believed to be associated with its ability to decrease the content of primary amines (norepinephrine, dopamine, and 5-hydroxytryptamine) in the central nervous system.

* Received August 21, 1959, from the College of Pharmacy, The Ohio State University, Columbus.

† Fellow of the American Foundation for Pharmaceutical Education, 1959–1960.

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The use of iproniazid, a monoamine oxidase inhibitor, as a tool for investigations in the area of brain chemistry has helped to clarify the mode by which reserpine may affect the function of the central nervous system. It is believed that it will elevate the content of primary amines in the brain (5). Combinations of iproniazid and reserpine can produce central stimulation which is characterized by an increase in sympathetic tone, tremors, and marked excitation very similar to an amphetamine-like action. This phenomenon is dependent upon the order in which iproniazid and reserpine are administrated and usually appears only when iproniazid is given before reserpine. Indeed, prior treatment with iproniazid prevents the stupor and depressed state produced by reserpine in mice and other animals (6). Iproniazid has been described as a psychic stimulant but, like reserpine, it will increase the sleeping time of barbiturates (7–9). It has been reported to have anticonvulsant properties and will increase the convulsive threshold of metrazol shock (10).

These effects indicate a complex interplay between central activity and the actions which characterize iproniazid and reserpine administration. The duration of action of each drug is long lasting, seemingly irreversible, and apparently noncompetitive with respect to the different possible mechanisms of action (11, 12). The activity of these drugs persists considerably beyond the time they can be detected in the tissues of the brain, blood, and other organs, indicating that they produce some unusual change (13).

The value of metrazol shock for the routine screening of anticonvulsant agents has been firmly established (14–17). As usually employed

this method is based on measuring the ability of a drug to raise or lower the chemoshock threshold rather than an alteration in the seizure pattern. Recently, Toman, *et al.* (18), evaluated the seizure latency interval in the convulsive shock pattern as a screening method for investigating antipsychotic drugs. A modification of this procedure using metrazol shock has been adapted to this problem.

The convulsive pattern of metrazol seen after a challenging dose given intraperitoneally can be divided into three stages as described by Orloff, *et al.* (14). First, a sharp twitching of the animal body which is followed by a series of clonic movements and then a stage of depression or a resting phase (seizure latency interval). Finally, a persistent convulsion occurs which consists of a tonic flexor component followed immediately by a usually lethal tonic extension of the hindlimbs. The three stages follow each other as the concentration of metrazol is gradually increased in the blood stream. The ability of a compound to prevent or facilitate the convulsion can be measured in terms of the final maximal extension as the end point and the latency time as the interval between clonic and tonic signs. Reserpine does not influence the sequence of events in this convulsive pattern. It will modify the resting stage, which is considered to be the time taken for selective high frequency stimulation of the motor cortex causing localized repetitive discharges to spread out to a generalized maximal tonic seizure. It could also be considered a period of inhibition of midbrain centers or other areas holding the medullary extensor facilitating center in abeyance (18). Central depressants and adrenergic stimulating drugs will generally suppress the tonic extensor phase; barbiturates and amphetamine-like agents, and anticonvulsants raise the threshold for this convulsive discharge. On the other hand, reserpine is found capable of facilitating the tonic component, reducing the convulsive threshold, and shortening the seizure latency time.

In view of the evidence that a relationship exists between the action of iproniazid and reserpine on the various biochemical changes produced by these drugs in the central nervous system especially when coupled with the many pharmacologic effects associated with their single and combined use, an investigation concerned with the effects of reserpine and iproniazid on the convulsive threshold, changes in facilitation, and in the seizure pattern of metrazol shock is reported and discussed.

EXPERIMENTAL

The experimental determinations were divided into three series depending on the drugs used (iproniazid, reserpine, and iproniazid-reserpine combinations), and was subdivided by various pretreatment schedules. Each series consisted of young adult female Swiss mice obtained from Maxfield farms weighing 20 ± 2 Gm. All animals were kept under uniform laboratory conditions and given free access to Purina chow and water. The animals were starved and housed in isolation cages for eight hours prior to metrazol shock. An experimental series consisted of 48 animals divided into four groups of 12 mice, all pretreated in a similar manner with iproniazid, reserpine, or a combination of both drugs. In each series for each pretreatment schedule the relative increase or decrease in the shock threshold was compared to a control-convulsive dose of metrazol obtained graphically by probit method using four dosage levels representing 12 animals per dose (19). This convulsive dose of metrazol is the index that will produce the tonic hindlimb extensor component of the seizure in 50% of the treated animals (CD_{50}). A 95% confidence limit is given with the CD_{50} of metrazol for each determination. For each group of 48 animals (four doses, 12 animals per dose) the seizure latency time was concurrently measured in those animals reaching the tonic extensor end point of the seizure pattern and was measured as the time, in seconds, between the clonic and maximal tonic phases. The mean time of those animals reaching the tonic extensor stage was calculated and a 95% confidence limit was statistically established using the standard error of this mean. The time interval between the administration of metrazol and the onset of the clonic stage was found to be appreciably uniform with the control. Facilitation was seen only in the series of animals pretreated with reserpine and was evaluated as the per cent of animals in which the resting phase was absent and the convulsion seizure passed directly from a clonic to a tonic phase in each determination.

The dose of iproniazid phosphate was 150 mg./Kg. given i. p. at various pretreatment intervals. This dose is nontoxic, produced few positive observable effects in mice after five days of daily administration, and was established as that dose which will inhibit monoamine oxidase and cause reserpine reversal.

The dose of reserpine was 8 mg./Kg. given i. p. at various pretreatment intervals with iproniazid. When given alone the dose was varied as indicated below.

The pretreatment schedule used with each drug or combination of drugs was based on reports by other investigators and partially on preliminary findings in our laboratory. Iproniazid (150 mg./Kg.) was injected at four, eight, twelve, and twenty-four hours, and daily for two, three, four, and five days prior to metrazol shock. In the latter grouping, shock was given twenty-four hours after the last treatment. Reserpine (8 mg./Kg.) was injected four and eight hours before shock; 4.0, 2.0, 1.0, 0.1 mg./Kg. was injected four hours before shock; and 0.1 mg./Kg. was given daily for ten days, the last dose being twenty-four hours before metrazol shock. Combinations of iproniazid (150 mg./Kg.) and reserpine (8 mg./Kg.) were injected at various in-

tervals before metrazol shock. These pretreatment combinations were iproniazid and reserpine together four hours before; reserpine (eight hours) and iproniazid (four hours) before; iproniazid (eight hours) and reserpine (four hours) before; iproniazid (twenty-four hours) and reserpine (four hours) before; iproniazid (daily for two days) and reserpine (four hours) before; iproniazid (daily for three days) and reserpine (four hours) before; iproniazid (daily for four days) and reserpine (four hours) before; iproniazid (daily for five days) and reserpine (four hours) before metrazol shock.

RESULTS AND DISCUSSION

This investigation is primarily concerned with the effects of iproniazid and reserpine on the convulsive threshold, facilitative action, and seizure latency time. It also compares the relative changes in the convulsive dose (CD_{50}) and the seizure pattern under similar pretreatment intervals and doses for iproniazid and reserpine. The results of this study are grouped according to the pretreatment schedule of drugs. Figure 1 indicates the responses seen with iproniazid pretreatment. Each set of adjacent bars represents two different parameters, the CD_{50} (mg./Kg.) and the seizure latency time (seconds). Figure 2 shows the effects seen with reserpine. The facilitative action of this drug is shown as the per cent of animals reaching the final tonic end point without showing the characteristic latency period between clonic and tonic phases of the convulsive seizure. Figure 3 represents the combined pretreatment with iproniazid and reserpine. In this figure three situations occurred where the facilitative action of reserpine was observed. The importance of this effect will be discussed below. The net result shown in Fig. 3 is that the optimal response in Fig. 2, e. g., reserpine (8.0 mg./Kg.) four hours, has been superimposed upon the effects of iproniazid seen in Fig. 1, making the various interactions of these drugs stand out as a function of the pretreatment interval which was used with each drug before metrazol shock.

In Fig. 1, pretreatment with iproniazid in single doses showed a moderate increase in the CD_{50} as the pretreatment interval was lengthened from four to twenty-four hours. This could be due to the effect of inhibiting the enzyme, monoamine oxidase, and the accompanying rise in the level of norepinephrine and other amines. Daily doses of iproniazid for two, three, four, and five days did not appreciably change the elevated convulsive threshold and the anticonvulsive effect. This would indicate the maximal action of this drug on metrazol shock was reached. The seizure latency time was similar or raised above the control index in all of the pretreatment periods. The gross effect was a general lengthening of the resting stage of the convulsive pattern with iproniazid pretreatment. In summary, the results of this series suggest that the convulsive dose and seizure latency time, while apparently unrelated, seem to provide a more accurate picture of the total anticonvulsant effect of iproniazid.

In Fig. 2 the effects of various doses of reserpine (0.1 to 8.0 mg./Kg.) using pretreatment intervals of four and eight hours and a ten-day period of daily doses (0.1 mg./Kg.) are shown together with the influence of this drug on the convulsive dose, facilitatory

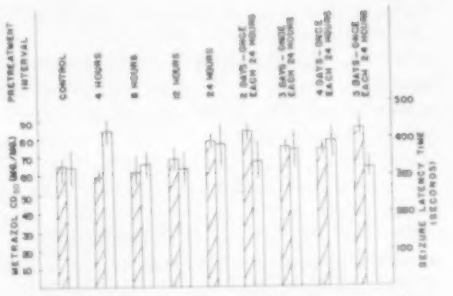

Fig. 1.—The effect of iproniazid on the convulsive dose and seizure latency time of metrazol. Pretreatment with iproniazid (150 mg./Kg.) CD_{50} ▽△, latency □.

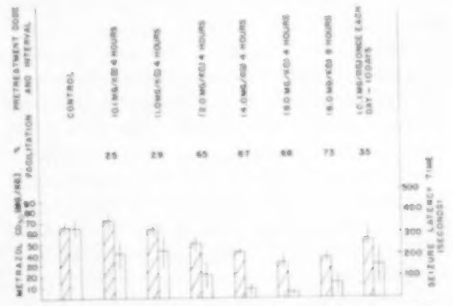

Fig. 2.—The effect of reserpine on the convulsive dose, seizure latency time, and facilitative action of metrazol. CD_{50} ▽△, latency □.

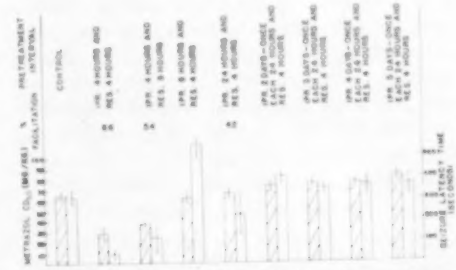

Fig. 3.—The effect of iproniazid and reserpine on the convulsive dose, seizure latency time, and facilitative action of metrazol. Pretreatment with iproniazid (150 mg./Kg.) and reserpine (8 mg./Kg.). CD_{50} ▽△, latency □.

action, and the seizure latency time. In sharp contrast to that of iproniazid, the relative change in the convulsive threshold and latency interval seems to depend on the change in the dose of reserpine. High doses (2.0 to 8.0 mg./Kg.) lowered the convulsive dose almost 50% and the seizure latency time dropped 90% below the control level. The results tend to show a correlationship between the dose of reserpine and the magnitude of the response, as can be observed by the steady fall in CD_{50} and latency time

as the dose of reserpine was increased from 0.1 to 8.0 mg./Kg. This relationship was also noticed in the proportion of animals showing no resting period, going from initial clonic to final tonic convulsion with increasing doses of reserpine. Extending the pretreatment interval or reducing the dose decreased the facilitating effect, raised the CD_{50}, and lengthened the latency time. Prolonged administration of 0.1 mg./Kg. of reserpine caused a moderate decrease in the CD_{50} and latency time with some increased facilitative action, perhaps due to the cumulative and prolonged effects of this drug. Summarizing the action of reserpine on metrazol shock; facilitation, latency time, and the convulsive threshold appear to be a function of the dose of reserpine, the former effect changing directly and the latter two changing inversely with the dose of reserpine. With this drug, the latency interval and the threshold of stimulation appear to complement each other as the amount of facilitation is increased. These findings with reserpine are in agreement with those of other investigators (20, 21).

In Fig. 3, the combined effect of iproniazid and reserpine is pictured as a predominating action of the drug which was administered first and the pretreatment interval between these drugs. Essentially, prior treatment with reserpine at four and eight hours gives much the same results noted in Fig. 2 for the same dosage and time. The anticonvulsant properties of iproniazid did not seem to reverse the low convulsive threshold, short latency time, and facilitatory action to any appreciable extent. It was observed that all the animals first treated with reserpine were characteristically depressed and remained in this state after iproniazid was given. In marked contrast to these findings are those seen with iproniazid treatment before reserpine. Reference to Fig. 1 shows a striking similarity between the prior treatment with iproniazid alone and when reserpine is given after iproniazid, as observed in Fig. 3. These animals were stimulated and active approximately a half hour after reserpine was added to their schedule. This adrenergic-type reaction was not apparent in those animals treated with iproniazid alone, as in Fig. 1. Consequently, an anticonvulsant effect was noted with the iproniazid-induced reversal of reserpine stupor. Apparently the protection by iproniazid was diminished after twenty-four hours as indicated by the lowered latency time and increased facilitative action. However, reinforcement with daily doses of iproniazid restored the anticonvulsant effect. The seizure latency time was lengthened and a general increase in the convulsive threshold was observed. The finding that iproniazid has anticonvulsant properties agrees with reports that various monoamine oxidase inhibitors, including iproniazid, protect against maximal shock seizures (22). The onset and duration of this agent seems to coincide with the reported rapid and persistent ability to increase brain levels of norepinephrine and 5-hydroxytryptamine (23).

Pretreatment with reserpine will lower the level of these amines. This would suggest that the anticonvulsant effect of iproniazid and reserpine combinations are the result of an increased level of amines caused by pretreatment with iproniazid before reserpine which prevents the destruction of those amines released by reserpine. It follows that the latent convulsive effect of reserpine is a reflection of the release, inactivation, and lowering of the amine level in the brain.

While our data do not directly support the relative importance of monoamine oxidase inhibition and the level of norepinephrine and other amines in the convulsive phenomenon, the results observed in this study on the convulsive dose, seizure latency time, and facilitating action of iproniazid and reserpine reflect many of the changes produced by these drugs. Although this would point to these biochemical factors as controlling the neurological mechanism, convulsive seizure and the related convulsive threshold it appears very unlikely that the anticonvulsant and convulsant properties of the many drugs used today all depend on the same factors.

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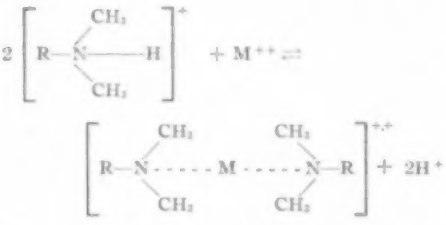
Metal Complexes of Tetracaine Hydrochloride and Related Local Anesthetics*

By HERBERT A. PLATT† and ALFRED N. MARTIN

The plan of this investigation was to complex tetracaine HCl and some related local anesthetics with certain metal ions; and to determine the most acceptable complexing metal ions, the stability constants of the metal complexes under varying conditions, and the biological significance of such complexes.

WITH THE POSSESSION of functional groups such as the secondary aromatic amine, the tertiary aliphatic amine, and the carboxyl group bound in the ester linkage, tetracaine has potential coordination centers.

The expectation was that tetracaine HCl should complex with suitable metal ions in the following manner:



This complexation is somewhat similar to that of metals with amino acids.

Since cations of the transition and near-transition metals have the greatest tendency for combining with electron donors (1), the following ions were selected: copper (II), nickel (II), cobalt (II), manganese (II), and zinc (II).

A rapid qualitative and quantitative method of testing for complexation is the pH effect. Qualitatively, the method is both quick and simple. A drop in pH results when the protonated form of the complexing agent forms a coordination compound with metal ions. The greater the tendency for the metal to combine with a given coordinating agent, the greater the drop in pH. Quantitatively, it is probably the most accurate and reliable method for the determination of the stability constants (2, 3).

EXPERIMENTAL

Materials.—Tetracaine HCl (U. S. P., Sterling-Winthrop Research Institute); procaine HCl (U. S. P., Mallinckrodt Chemical Works); butethamine HCl (N. F., Novocol Chem. Mfg. Co.); glycine (General Biochemicals Inc.); DL-α-alanine (General Biochemical Co.); β-alanine (General Biochemicals Inc.); and DL-serine (Nutritional Biochemicals Co.) were evaluated chromatographically and were found to be of a high degree of purity and could be used without prior treatment.

Analytical reagent grades of the metal chlorides were satisfactory and were used without further purification.

The local anesthetics, procaine HCl and butethamine HCl, in addition to tetracaine HCl, provided a parallel in the relationship between the stability constants and the structure of the local anesthetics.

The amino acids were selected to serve as reference compounds for the procedures developed and to provide a comparison between the stability constants and the structure.

Procedure.—Using the primary standard, potassium acid phthalate, standard sodium hydroxide solutions of approximately 0.2 *N* were prepared as needed in the usual quantitative manner.

The following is a general outline of the procedure used: a ligand hydrochloride or amino acid stock solution was freshly prepared containing approximately 5.0×10^{-4} moles/ml. Five milliliters, by pipet, of this stock solution (2.5×10^{-3} moles) was mixed with sufficient distilled water to give 75 ml. and was titrated with the standard sodium hydroxide (0.50-ml. increments), by buret. The pH, at each addition of the base, was determined with a Beckman model GS pH meter with fiber-type calomel reference and glass (heavy duty) electrodes. One milliliter, by pipet, of the metal stock solution (1 to 10×10^{-4} moles), also diluted to 75 ml., was titrated with sodium hydroxide.

To test for complexation with the metal, 1.00 ml. of the metal stock solution was added to 5 ml. of the ligand stock solution (all samples were diluted to 75 ml.) and the resulting solutions were titrated as outlined. In certain cases, to prevent hydroxide formation before the titration could be carried out (zinc), the titrated samples were previously acidified by the addition of 5.00 ml. of approximately 0.01 *N* HCl. The titrations were conducted in a water bath at 30° ±0.5°; the solutions were allowed to reach this temperature for fifteen to twenty minutes prior to the titration. During the titration, the solution was agitated by a slightly flattened glass stirring rod driven by an air stirrer. The agitation was not severe enough

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† Present address: Julius Schmid, Inc., New York, N. Y.
The authors are indebted to W. H. Golod for his technical assistance in the biological portion of this study.

to break the surface of the liquid. The pH meter was standardized with a buffer solution of pH 7.00. All values reported are an average of two separate determinations. The precision of the pH measurements was ±0.02 pH unit.

All calculations are based on a constant volume of 75 ml. Disregarding the volume changes, this led to an estimated error of 2% in addition to the experimental error of about 2%.

Treatment of Data.—*Qualitative.*—Applying Bjerrum's method (4) to a case of equilibrium between metal ions and a complexing agent, the following examples are presented for the pH titration of glycine (Fig. 1) and tetracaine HCl (Fig. 2) in the presence and absence of cupric ions. It was not found necessary to continue the titration to the equivalence point; each curve was carried approximately to the half-neutralization point.

Curve I, in Figs. 1 and 2, shows the titration curve of a 75-ml. sample of ligand. Curve II shows the titration curve of a 75-ml. sample of the ligand to which a smaller quantity of cupric ion was added. The curve with the metal lies below the ligand curve indicating that complexation has taken place. The first flat portion of Curve II represents the formation of the complex, the sharp rise indicates the completion of the complex formation, and the last flat portion represents the titration of the excess ligand.

Calculation of Stability Constants.—Using the method of Bjerrum (4), as modified by Calvin and Melchior (5), $\bar{n}$, the average number of ligand molecules bound to a single metal ion, may be evaluated from the horizontal distance between titration curves I and II, Figs. 1 and 2. This is exactly the amount of base consumed in the reactions, and equals the concentration of BH^+ bound in the complex

$$M^{+2} + BH^+ + OH^- = (MB)^{+2} + H_2O$$

$$(MB)^{+2} + BH^+ + OH^- = (MB_2)^{+2} + H_2O$$

and the overall reaction is

$$M^{+2} + 2BH^+ + 2OH^- = (MB_2)^{+2} + 2H_2O$$

where M^{+2} represents the metal ion, BH^+ is the protonated ligand, and $(MB)^{+2}$ and $(MB_2)^{+2}$ are complexed forms of the ligand. It is assumed that these are essentially the only reactions resulting from the uptake of base since the complexing agent is present in considerable excess with respect to the metal ion. The total moles of the ligand B bound, equivalent to the horizontal distance between curves I and II, when divided by the total concentration of metal, gives the value of $\bar{n}$. When $\bar{n}$ becomes constant, the maximum for the average number of ligand molecules bound to a single metal ion is had. At any pH, the value of unbound or free B is calculated from $p(B) = -\log B = pKa - pH - \log [(BH^+)_{initial} - (NaOH)]$. In doing so, a series of values of $\bar{n}$ and B are obtained (6). Figures 3 and 4 are graphical representations of $\bar{n}$ plotted against the negative logarithm of B, written p(B). Approximate values for $^1/_2 \log K$, where log K is the overall stability constant, may be read directly from these formation curves at $\bar{n} = 1.0$. The data are shown in Table I.

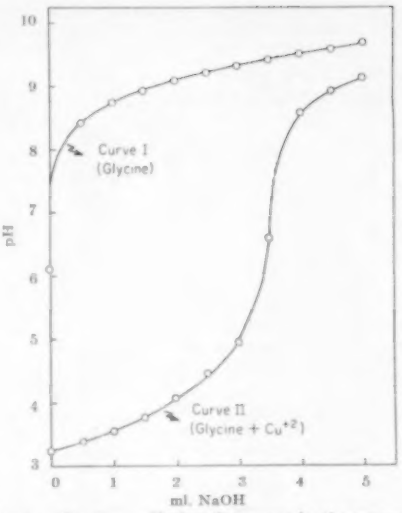


Fig. 1.—Glycine; pH titration curve in the presence and absence of cupric ions.

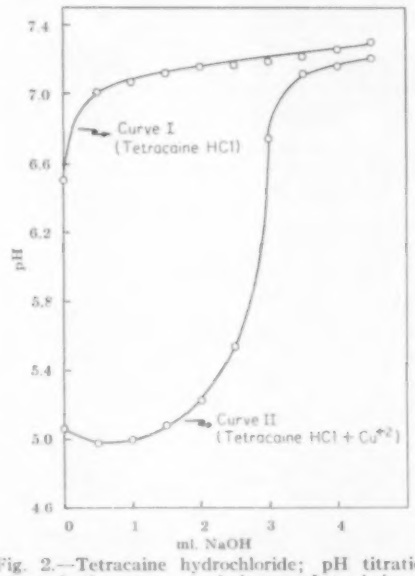


Fig. 2.—Tetracaine hydrochloride; pH titration curve in the presence and absence of cupric ions.

RESULTS

Qualitatively, cobalt, manganese, and zinc ions did not complex with tetracaine HCl since no pH lowering was noted when tetracaine HCl was titrated in the presence of these metal ions. Nickel complexed to a very limited extent with the ligand, tetracaine.

Copper showed a tendency toward complexation with tetracaine. Since the copper-tetracaine titra-

TABLE I.—TYPICAL CALCULATION OF $\bar{n}$ AND THE STABILITY CONSTANT

pH	H_2O^+	NaOH, ml.	Moles OH^- = moles B (bound)	$\bar{n}$	(B)	p(B)
Glycine, 30°, 75-ml. sample, 3.33 × 10^{-2} moles/L.; Cu^{+2}, 6.06 × 10^{-3} moles/L.; NaOH, 2.59 × 10^{-4} moles/ml., 0.50-ml. increment						
3.50	3.16×10^{-4}	0.98	2.53×10^{-4}	0.56	1.95×10^{-3}	7.71
4.00	1.00×10^{-4}	1.88	4.86×10^{-4}	1.07	5.50×10^{-5}	7.26
4.50	3.16×10^{-5}	2.52	6.53×10^{-4}	1.43	1.59×10^{-7}	6.80
5.00	1.00×10^{-5}	3.02	7.83×10^{-4}	1.72	4.68×10^{-7}	6.33
5.50	3.16×10^{-6}	3.27	8.48×10^{-4}	1.86	1.41×10^{-6}	5.85
6.00	1.00×10^{-6}	3.40	8.82×10^{-4}	1.93	4.36×10^{-6}	5.36
6.50	3.16×10^{-7}	3.48	9.02×10^{-4}	1.98	1.38×10^{-5}	4.86
7.00	1.00×10^{-7}	3.52	9.13×10^{-4}	2.00	4.36×10^{-5}	4.36
7.50	3.16×10^{-8}	3.52	9.13×10^{-4}	2.00	1.38×10^{-4}	3.86
8.00	1.00×10^{-8}	3.52	9.13×10^{-4}	2.00	4.36×10^{-4}	3.36

From Fig. 3, $^1/_2$ log K was found to be 7.35, and log K = 14.70. The value, $\bar{n}$ = 2.00, is a maximum for the average number of ligand molecules bound to a single metal ion.

Tetracaine HCl, 30°. 75-ml. sample, 3.34 × 10^{-2} moles/L.; Cu^{+2}, 6.06 × 10^{-3} moles/L.; NaOH, 2.48 × 10^{-4} moles/ml., 0.50-ml. increment						
5.10	7.94×10^{-4}	1.60	3.97×10^{-4}	0.87	1.51×10^{-5}	4.82
5.20	6.31×10^{-6}	1.95	4.84×10^{-4}	1.06	1.82×10^{-5}	4.74
5.40	3.98×10^{-6}	2.30	5.71×10^{-4}	1.25	2.76×10^{-5}	4.56
5.60	2.51×10^{-6}	2.55	6.32×10^{-4}	1.39	4.26×10^{-5}	4.37
5.80	1.58×10^{-6}	2.70	6.69×10^{-4}	1.47	6.70×10^{-5}	4.18
6.00	1.00×10^{-6}	2.85	7.07×10^{-4}	1.55	1.02×10^{-4}	3.99
6.20	6.31×10^{-7}	2.90	7.19×10^{-4}	1.58	1.62×10^{-4}	3.79
6.40	3.98×10^{-7}	2.95	7.31×10^{-4}	1.60	2.51×10^{-4}	3.60
6.60	2.51×10^{-7}	2.95	7.31×10^{-4}	1.60	3.93×10^{-4}	3.40

From Fig. 4, $^1/_2$ log K was found to be 4.77 and log K = 9.54.

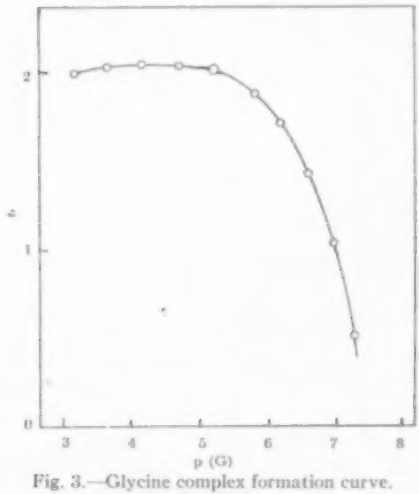


Fig. 3.—Glycine complex formation curve.

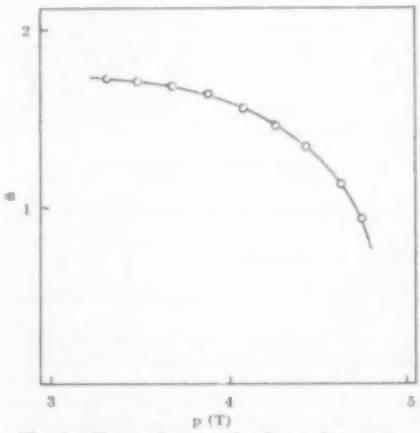


Fig. 4.—Tetracaine complex formation curve.

tion samples become slightly opalescent in the near neutral range, the cupric ions were probably not tightly bound to the tetracaine molecule and were being removed from solution through the formation of hydroxo complexes and hydroxides. Therefore, it is reasonable to assume that the complex formation was limited by the formation and solubility of cupric hydroxides.

Compounds having a structure similar to that of the tetracaine salt were also tested for their ability to complex with the cupric ion; it was found that the hydrochloride salts of both procaine and butethamine formed complexes with copper.

Determination of Stability Constants.—Tetracaine HCl, procaine HCl, butethamine HCl, glycine, α-alanine, β-alanine, and serine, with and without cupric ions, were prepared and titrated as outlined. The procedure was then repeated, varying the concentrations of the metal ion.

The apparent acidity (dissociation) constants of the complexing agents, as defined by Brönsted (7), were determined and are tabulated in Table II. The concentration range of ligands varied from 1.25 to 2.50 × 10^{-3} moles/sample; therefore, the ionic strength of the samples was considered negligible. The literature values of the pK'a were corrected to 30° by means of the correction factors given by Albert (10).

TABLE II.—APPARENT ACIDITY CONSTANTS OF THE COMPLEXING AGENTS[a]

Compound	pK'a (Found) [b]	pK'a (Literature)	Reference
Glycine	9.69	9.71	(8)
α-Alanine	9.86	9.80	(8)
β-Alanine	10.45	10.30	(8)
Serine	9.23	9.26	(8)
Tetracaine HCl	8.37	8.33	(9)
Tetracaine HCl[c]	8.59	...	...
Procaine HCl	8.99	9.05	(9)
Monocaine HCl	9.02	...	...

[a] Comparison between results of this investigation and literature values corrected to 30°.
[b] At ionic strengths of approximately 0.03, except for [c].
[c] At an ionic strength of approximately 0.67.

TABLE III.—STABILITY CONSTANTS OF COPPER WITH VARIOUS LIGANDS[a]

Copper Complex	ñ	log K Found	log K Literature [b] (11)
Glycine	2.00	14.7	15.0
α-Alanine	2.03	15.0	14.8
β-Alanine	2.02	12.4	12.6
Serine	2.09	15.2	14.4
Tetracaine	1.71	9.6	...
Tetracaine[c]	1.52	9.8	...
Procaine	1.53	10.6	...
Monocaine	1.76	10.9	...

[a] At ionic strengths of approximately 0.03, except for [b] and [c].
[b] Literature value at a low ionic strength (*ca.* 0.01).
[c] At an ionic strength of approximately 0.67.

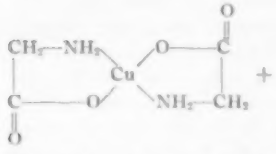
Using the method previously described, the p(B) and ñ values were computed. The calculation was repeated for each concentration of cupric ion used, and the stability constant was obtained from the formation curve. Table III presents these values. The binding of the copper to the drugs and amino acids would appear to be of the type previously postulated.

The Binding of Tetracaine HCl to Copper-Amino Acid Complexes.—The objectives of this portion of the investigation were (*a*) to determine the possible direct complexation of tetracaine HCl with amino acids, and (*b*) to determine the possible mediation of binding by metal ions, as Klotz and Loh Ming (12) have done.

Samples of the amino acid-copper complexes, glycine, α-alanine, β-alanine, and serine were prepared stoichiometrically in a 2:1 ratio and were titrated with sodium hydroxide in the presence of tetracaine HCl, as was previously outlined.

It was found that the amino acids did not complex directly with tetracaine HCl. The second possibility, mediation binding by the metal ion, was indicated since the pH values of the samples of the three components, tetracaine HCl + amino acid + cupric ion were lower than in any of the other solutions. Using the method for the calculation of the stability constant, as previously outlined, the stability constants and ñ were calculated (considering the amino acid-copper complex as the 'metal ion'). Table IV is a tabulation of the results obtained with the four amino acids.

Ley (13) has shown that the $Cu(Glycine)_2$ complex may be altered by the addition of a foreign ligand. In this alteration the chelate rings of the original compound are opened to yield a complex. Therefore, the reaction that occurs between $Cu(Glycine)_2$ and tetracaine may be of the type:



$$2\,TH^+ \rightleftharpoons [Cu(T)_2(OOC{-}CH_2{-}NH_2)_2] + 2H^+$$

The variation that was evident with β-alanine, may be due to the greater distance that exists between the amino and carboxyl groups.

TABLE IV.—STABILITY CONSTANTS FOR THE BINDING OF TETRACAINE HCL TO COPPER-AMINO ACID COMPLEXES

Copper-Amino Acid Complex	ñ	log K
Copper-glycine	1.90	10.3
Copper-α-alanine	2.20	10.3
Copper-β-alanine	1.30	7.7
Copper-serine	2.00	10.3

Relationship Between pK'a and log K.—A rough proportionality has been found to exist between the basic strength of amines and their complex forming affinity (14, 15, 16). Martell and Calvin (2) state that, in general, characteristics of the complex formation are a correlation with the basic strength of the electron donor and the effect of substitution on the donor.

Accordingly, the degree of binding (stability constant) should be directly related to the dissociation constant (pK'a) of the drug as, in fact, was found as shown in Fig. 5.

It is well established that trace metals often play a part in drug mechanism. An excess of the metal ions can serve to decrease drug action substantially by binding with the key dissociating functional groups. On the other hand, an excess of the trace metals may allow the drug to elicit a greater effect at its site of action. The log K of their copper

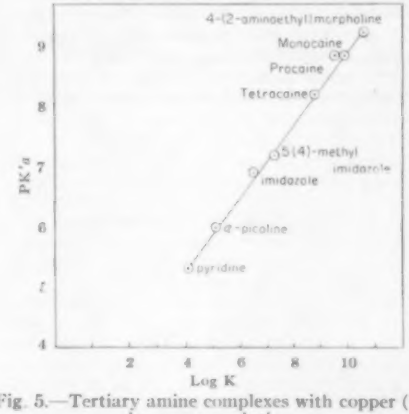


Fig. 5.—Tertiary amine complexes with copper (II) in aqueous solution.

complexes of the tertiary amines may, therefore, be related roughly to the physiological action of these agents. Accordingly, the knowledge of the physical properties and physico-chemical constants provides an avenue of approach to drug mechanism that could well lead to a more successful prediction of biological activity, both qualitative and quantitative, as well as to give an estimate of the selective toxicity of new compounds prior to pharmacological screening.

A Biological Evaluation of the Copper-Tetracaine Complex.—The objective of this part of the study was to determine whether or not the complexed form of tetracaine HCl affected the local anesthetic activity of tetracaine HCl. Specifically, tetracaine HCl and tetracaine HCl with varying copper concentration solutions were tested by means of corneal anesthesia and infiltration anesthesia in the guinea pig to determine any differences in the duration of action and degree of anesthesia.

The guinea pig cornea and intradermal wheal methods were selected for comparing the degree and durations of action of the solutions (17, 18, 19). Both tests are simple, rapid, and sensitive.

For the cornea anesthesia test a 1.00% tetracaine HCl solution (solution 1) was prepared. The various concentrations of cupric ion were so selected to remove, theoretically, 21% (solution 2), 39% (solution 3), and 100% (solution 4) of the tetracaine HCl.

For the wheal test, a tetracaine HCl concentration of 0.05% was chosen for comparing the effectiveness of the complexed forms. The concentrations used in the corneal method were diluted 20 times with distilled water to a 0.05% concentration. In both cases, all solutions were freshly prepared before use, and the animals and solutions were randomly selected. The investigator was unaware of the identities of the solutions until the experiments were completed. A statistical evaluation of the results is found in Table V.

In the corneal method, no significant statistical differences were noted between samples, as compared to solution 1. In the wheal method, no statistical differences were noted with solutions 2 and 3, as compared with solution 1. However, statistical significance was noted with solution 4 since this result would be had more than 95% of the time. This would indicate that the presence of cupric ions in tetracaine HCl decreased the degree of local anesthesia to a slight extent. It would appear that the presence of cupric ions immobilized the aliphatic-type amine portion of the local anesthetic that is thought to exert the sensory anesthesia (9). This would support the previously postulated mechanism of complexation.

TABLE V.—A STATISTICAL EVALUATION OF THE BIOLOGICAL DATA

Solution	*t* Test	Std. Deviation	Std. Error of Mean	Mean Values
	Corneal Method[a]			
1	...	2.83	1.00	12.63
2	$0.537 > p > 0.5$	3.69	1.30	11.75
3	$0.400 > p > 0.5$	3.36	1.19	13.25
4	$0.391 > p > 0.5$	4.65	1.64	11.88
	Wheal Method			
1	...	6.92	2.45	6.75
2	$1.470 < 0.2 \quad p > 0.1$	10.72	3.78	13.38
3	$1.003 < 0.4 \quad p > 0.3$	8.50	3.00	10.63
4	$2.197 < 0.05 \quad p > 0.02$	14.00	4.95	18.88

[a] Using units of 5 min.

An evaluation of the copper-glycine-tetracaine complex by the corneal anesthesia test showed no statistically significant differences between the complexed samples and solution 1.

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The Application of $\Delta\epsilon$-Analysis to Pharmaceuticals: The Determination of Eugenol*

By JULIUS C. DEMETRIUS, Jr.†, and JOSEPH E. SINSHEIMER

The $\Delta\epsilon$-method of analysis, which was developed for the investigation of lignins, involves the selective modification of a given chromophore in a mixture of chromophores. This method permits the quantitative determination of a single ultraviolet-absorbing compound in mixtures of such absorbing materials. The analyses of eugenol in clove oil, in eugenyl acetate, and in two formulations are described as illustrations of the pharmaceutical application of this technique. The absorbance at 296 mμ of an aliquot of the eugenol-containing preparation was determined in basic solution. The change in absorbance was then compared to the bathochromic displacement of a standard eugenol solution with a corresponding change in pH.

THE STUDY of the phenolic compounds of various lignins by Aulin-Erdtman (1, 2) and by Goldschmid (3, 4) was based in part upon the development of "$\Delta\epsilon$-curves." In these investigations the $\Delta\epsilon$-curves were obtained by subtracting the absorbances of the U. V. spectra of lignin derivatives in solution of low pH from their corresponding spectra in solution of high pH. By comparing $\Delta\epsilon$-curves obtained in this manner to those obtained from model compounds in a similar manner, both qualitative and quantitative studies of phenolic compounds, even in the presence of other U. V. absorbing material, were possible.

The method need not be limited to the bathochromic displacement of phenols in alkaline solution. Aulin-Erdtman (5) in a review article points out that the $\Delta\epsilon$-method may be applied whenever the absorption properties of a given chromophore can be modified selectively in the presence of a mixture of chromophores. A $\Delta\epsilon$-curve is still obtained by subtracting the spectrum of the starting material from the spectrum of the product. The absorbance of any non-modified chromophores is thereby cancelled out. Thus, in those cases where selective modification of the chromophore can be accomplished, $\Delta\epsilon$-analysis has the advantage of the analysis of a single absorbing material in a mixture of absorbing materials without the necessity for prior separation.

This technique should have wide application to pharmaceutical determinations. It is the purpose of this investigation to apply $\Delta\epsilon$-analysis to a quantitative pharmaceutical problem. The lignin studies involved eugenol as one of the model compounds. A direct pharmaceutical extension of these studies was undertaken in the assay of clove oil for its eugenol content and the assay of eugenol in a pharmaceutical product. In addition, the procedure developed for the assay of clove oil was tested by its application to a sample of eugenyl acetate.

EXPERIMENTAL

Ultraviolet absorption spectra and quantitative measurements were made with a Beckman, model DU spectrophotometer. All quantitative measurements were the average of six determinations. After the first three determinations, the cells containing the sample solution and blank solution were interchanged.

Materials.—Eugenol (Eastman Kodak Co.) was fractionated by vacuum distillation through a helices-packed column. The refractive index of the purified sample was $n_D^{20} = 1.5410$. Eugenyl acetate (Aldrich Chemical Co.) was fractionated by a similar distillation and crystallized from alcohol-water. The melting point of the purified sample was 30–31°. Clove oil U. S. P. (Magnus, Mabee and Reynard, Inc.) had a total phenolic content of 87% as determined by the U. S. P. XV assay. All other materials were of C. P. grade and were used without further purification.

Determination of Eugenol Spectra.—An accurately weighed sample of 59 mg. of eugenol was diluted with 50% alcohol-water to 250 ml. in a volumetric flask. A series of 10-ml. portions of this solution was transferred to 100-ml. volumetric flasks and diluted to the mark with a series of buffer solutions prepared from standard solutions of sodium hydroxide and sulfuric acid. The pH of each of these eugenol solutions was measured by a Beckman Zeromatic pH meter and its U. V. spectrum determined. In this manner eleven spectra of eugenol in solutions ranging from pH 2.5 to 13.5 were obtained.

The Determination of $\Delta\epsilon$ for Eugenol.—Samples of about 50 mg. of eugenol were accurately weighed and diluted with alcohol in a 250-ml. volumetric flask. One 10-ml. portion of this solution was diluted with water and 1 ml. of 1.000 *N* sodium hydroxide in a 100-ml. volumetric flask. A second 10-ml. aliquot of the alcoholic eugenol solution was transferred to a 100-ml. volumetric flask and diluted with water and 1 ml. of 0.100 *N* sulfuric acid. The absorbance of the alkaline solution was determined relative to the acid solution in the ref-

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† Present address: Mead Johnson and Co., Evansville, Ind.

erence cell at 296 mμ. Blank solutions were also prepared, and any relative absorbance was determined in a like manner in order to correct for any contribution due to solvents and reagents. Seven such determinations were made. The alkaline solutions were recorded with a pH of 12.0 ± 0.2 and the acid solutions with a pH of 3.0 ± 0.2.

The Determination of Eugenol in Clove Oil and in Eugenyl Acetate.—Samples of about 60 mg. were accurately weighed and placed together with 3 ml. of 1 *N* sodium hydroxide and 15 ml. of alcohol in a 250-ml. volumetric flask. After the mixture was shaken for five minutes at room temperature, it was heated in a boiling water bath for fifteen minutes. During this time the flasks were shaken at five-minute intervals. The flasks were cooled to room temperature and alcohol was added to 250 ml. At the same time a control solution without sample was also prepared in this manner. Ten-milliliter aliquots of the saponified samples and the control were then diluted and buffered; the relative absorbances at 296 mμ were determined essentially as described under the determination of $\Delta\epsilon$ for eugenol. The only modification required was the addition of 2 ml. of 0.100 *N* sulfuric acid to obtain the desired pH of 3.0 ± 0.2 for the reference solution.

The Determination of Eugenol in Formulations.—An aliquot of the formulation was chosen to obtain a concentration of about 2 mg. per 100 ml. of eugenol in the reference and alkaline solutions. The procedure described under $\Delta\epsilon$ for eugenol was then followed.

Calculations and Definitions.—$\Delta\epsilon$ for eugenol: $\Delta\epsilon = (\Delta A \times 164.2)/c$

Percentage of eugenol: $\% = (\Delta A \times 164.2 \times 100)/(c \times \Delta\epsilon)$

Concentration: $c = (\Delta A \times 164.2)/\Delta\epsilon$

Where: $\Delta\epsilon$ = absorbance at 296 mμ of a 1*M* solution of eugenol in base less the absorbance of a 1*M* solution in acid. ΔA = the observed absorbance at 296 mμ of a given concentration of eugenol in basic solution less the absorbance of same concentration of eugenol in acid solution. c = concentration in Gm./L.

RESULTS AND DISCUSSION

Figure 1 represents the spectra of eugenol in (*a*) acid to neutral solution and (*b*) in alkaline solution. As is typical of phenols, there is a pronounced bathochromic displacement of both the E and B bands and an increase in the intensity of absorption of these bands. When the acid spectra is subtracted from that of the alkaline spectra, the $\Delta\epsilon$-curve illustrated in Fig. 2 is obtained.

The same $\Delta\epsilon$-curve is produced even in the presence of other U. V.-absorbing compounds provided that these compounds show no response to changes in pH. $\Delta\epsilon$-curves can be used in a manner analogous to normal U. V. curves for qualitative and quantitative purposes. Thus, the maxima at 246 and 296 mμ should serve as the basis for quantitative measurements.

It appears that the 246 mμ maximum, because of its greater intensity, would be the most useful for quantitative measurements. However, in actual

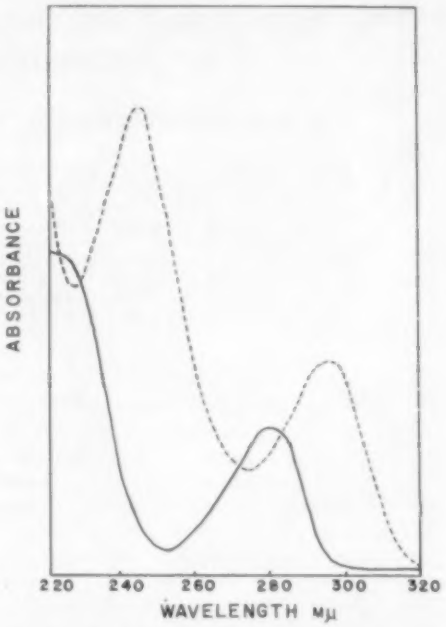

Fig. 1.—Ultraviolet absorbance spectra of eugenol; —— pH 7.0; - - - - pH 12.3.

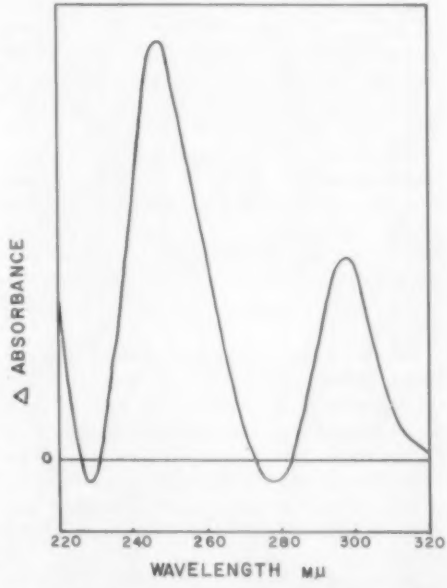

Fig. 2.—$\Delta\epsilon$ ultraviolet absorbance spectrum of eugenol at pH 12.3 with the eugenol spectrum at pH 7.0 as a reference.

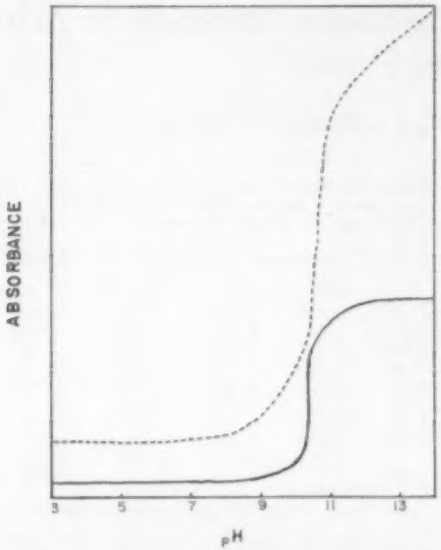

Fig. 3.—Maximum absorbance of eugenol as a function of pH; —— maximum at 296 mμ; - - - - maximum at 246 mμ.

practice this is not the case, since the 296 mμ maxima showed greater stability with changes in alkaline pH than did the 246 mμ maxima and, therefore, would require a less critical control of pH for quantitative comparisons. The change in absorbance of these peaks as a function of change in pH is illustrated in Fig. 3.

To serve as a basis for the quantitative comparison of eugenol solutions, the $\Delta\epsilon$ for the 296 mμ maxima was determined from standard solutions. The average of seven determinations gave a value of 3,886 for a molar solution of eugenol of a pH of 12.0 ± 0.2 over that of a molar solution of a pH of 3.0 ± 0.2 at 296 mμ.

Table I is a summary of the results of the determination of the percentage of eugenol in a sample of clove oil based upon this value for $\Delta\epsilon$. As a partial test of the procedure there is also included in Table I the results of the analysis of a sample of eugenyl acetate.

In order to investigate the application of $\Delta\epsilon$-analysis to a pharmaceutical formulation the following mouth wash, formula A, was compounded.

Saccharin, soluble	0.025 Gm.
Fuchsin, basic	0.005 Gm.
Peppermint oil	0.100 Gm.
Eugenol	0.100 Gm.
Alcohol	75.00 cc.
Distilled water to	250.00 cc.

Table II presents the results of the analysis of this solution for its eugenol content.

TABLE I.—ANALYSIS OF EUGENOL IN CLOVE OIL AND EUGENYL ACETATE

Sample	Clove Oil, %	Eugenyl Acetate, % (79.6% Calcd.)
1	85.4	80.0
2	83.8	79.9
3	85.7	81.2
4	86.7	79.5
5	86.0	...
6	84.9	...
7	85.8	...
Av.	85.5	80.2

TABLE II.—ANALYSIS OF EUGENOL IN MOUTH WASH FORMULAS

	Formula A, Gm.	Formula B, Gm.
Amount present	0.1004	0.1124
Found 1	0.0980	0.1090
2	0.0980	0.1107
3	0.0997	0.1104
4	0.0976	0.1101
5	0.1001	0.1094
6	0.0972	0.1103
Av.	0.0984	0.1099

The above formula was also modified by reducing the peppermint oil to 0.06 Gm. and including 0.06 Gm. of cassia oil which possesses U. V. absorption of high intensity. The results for the analysis of eugenol in the modified formula, formula B, are also presented in Table II.

It should be noted that the U. V. spectrum of cassia oil shows a limited sensitivity to changes in pH, and results recorded in Table II would have been about 10% lower unless corrected for this change. That is, the analysis of formula B required that the absorbance of a control solution without eugenol also be determined and be applied as a correction to the analysis of the complete formula. Formula B, therefore, demonstrates a limitation of $\Delta\epsilon$-analysis: the sensitivity of the other compounds in a mixture to the conditions used to modify the chromophore of interest. This limitation can be overcome in the analysis of dosage forms by running a control as noted in the analysis of formula B or by applying a simultaneous equation determination to the $\Delta\epsilon$-curve. If such corrections cannot be applied as, for example, in the assay of some natural products, the extent of these errors can readily be determined and, to a great degree, corrected by comparing the complete $\Delta\epsilon$-spectrum of mixture to that of the standard compound.

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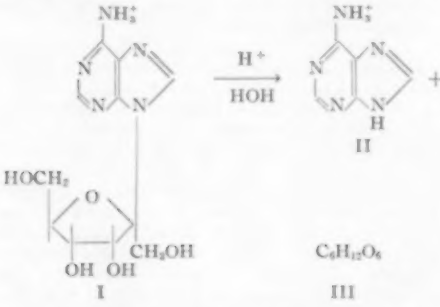
Psicofuranine: Correlation of Assay Methods in Acid Degradation Studies*

By EDWARD R. GARRETT and LADISLAV J. HANKA

The unnatural nucleoside, psicofuranine, is bimolecularly hydrolyzed by acid to adenine and the sugar psicose. Adenine appears to reverse the biological activity of psicofuranine in the plate-disk method against *S. aureus* so that standard curves must be prepared with the same amount of adenine as the material to be assayed. When this phenomenon is accounted for, the chemical and biological assays are shown to correlate. The kinetic constants for the acid catalyzed hydrolysis of the nucleoside have been determined.

THE NEW ANTIBIOTIC, psicofuranine (1), has been assigned the structure 6-amino-9-D-psicofuranosylpurine by Schroeder and Hoeksema (2). This structure has also been proposed for angustmycin C by Hsu (3). Properties and assay methods have been studied (4–14).

The purpose of this paper is to consider the acid catalyzed degradation of psicofuranine, I, by hydrolysis to adenine, II, and psicose, III;



as measured by both biological (6, 12) and chemical (10, 11) assays, and to determine the significance of the correlation between the assay methods.

EXPERIMENTAL

Kinetics of the *In Vitro* Degradation of Psicofuranine by Acid at 40°.—Sufficient psicofuranine, generally 25 mg., previously dried at 60° under high vacuum for forty-eight hours, was weighed into a tared 25-ml. volumetric flask, and made up to volume with the solvent of the appropriate hydrochloric acid concentration (see Table I). This solvent had been previously equilibrated in the 40° constant temperature bath. The resultant solutions were placed in the 40° temperature bath and at recorded time intervals assayed by the procedure of Forist (11) which has been applied to the chemical determination of psicofuranine in blood plasma and serum (10). Prior to the assay a 1-ml. aliquot was pipetted into a 5-ml. volumetric flask containing sufficient NaOH to slightly over-neutralize the aliquot so as to halt the acid degradation. This solution was then diluted with water up to volume and the assay procedure applied.

TABLE I.—RATE CONSTANTS FOR THE ACID HYDROLYSIS OF 1 MG./ML.[a] PSICOFURANINE AT 40° AS A FUNCTION OF HYDROCHLORIC ACID CONCENTRATION

Runs	10^4k (sec.$^{-1}$)	$[HCl]_A$	pH	Temp., °C.[b]
1	0.538	0.010	2.13	40.0
2	1.58	0.025	1.65	40.2
3	3.89	0.050	1.36	40.2
4	6.20	0.075	1.20	40.2
5	9.32	0.100	1.06	40.2
6[c]	9.14	0.100	1.05	39.3
7[c]	8.98	0.100	1.06	39.5
8[c]	8.21	0.100	1.05	39.2
9[d]	9.17	0.100	1.05	39.4
10[d]	9.17	0.100	1.05	39.4

[a] Except for run 6 at 0.42 mg./ml. and run 7 at 0.61 mg./ml. [b] Accurate within a run to ±0.05°. [c] Correlated with bioactivity; no apparent reason for low estimate of rate. [d] Correlated with bioactivity and run simultaneously except that an initially equimolar amount of adenine was added to run 10.

The rate of acid degradation of psicofuranine should be bimolecular, proportional to the psicofuranine molarity, [P], and the molarity of the hydrochloric acid. The apparent molarity, $[HCl]_A$, should be corrected for the amount of psicofuranine of pKa′ 3.9, determined by the pH at half-neutralization, which provides a true molarity of $[HCl]_A - [P]_o = [HCl]$ where $[P]_o$ is the initial concentration of the antibiotic. The pKa′ of the hydrolysis product adenine is almost identical so that the acid concentration will not vary throughout the reaction. Thus

$$-d[P]/dt = k_{H^+}\{[HCl]_A - [P]_o\}[P] \quad \text{(Eq. 1)}$$

Equation 1 may be integrated as a pseudo first-order equation so that

$$\log[P] = -kt/2.303 + \log[P]_o \quad \text{(Eq. 2)}$$

where

$$k = k_{H^+}\{[HCl]_A - [P]_o\} \quad \text{(Eq. 3)}$$

Typical plots according to Eq. 2 are given in Fig. 1 where the pseudo first-order nature of the acid degradation is confirmed by the linearity of the plots. The logarithm of the assayed absorbance which is proportional to the psicofuranine concentra-

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Grateful acknowledgments are given to (Mrs.) Lillian G. Snyder for excellent technical assistance, to Mr. Ernest Markovich for the operation of the digital computer in statistical correlations, and to Dr. Arlington A. Forist for helpful advice and discussions on the use of the chemical assay.

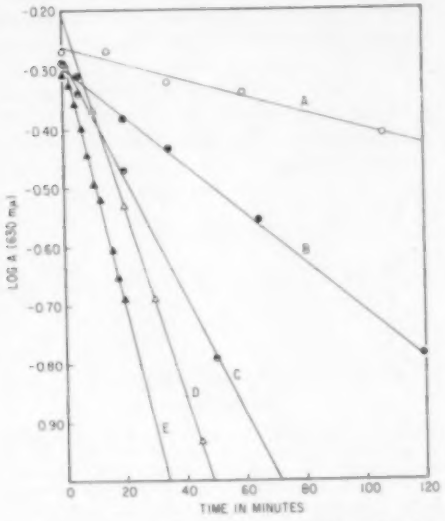

Fig. 1.—The pseudo first-order plots of psicofuranine degradations at 40° in varying molarities of HCl at 40.0°. The curves, HCl molarity and apparent pH are: *A*, 0.01 *M*, 2.13; *B*, 0.025 *M*, 1.65; *C*, 0.050 *M*, 1.36; *D*, 0.075 *M*, 1.20; *E*, 0.10 *M*, 1.05. The absorbance values, *A*, at 630 mμ, are proportional to psicofuranine content and are the readings determined by the assay procedure of Forist (11).

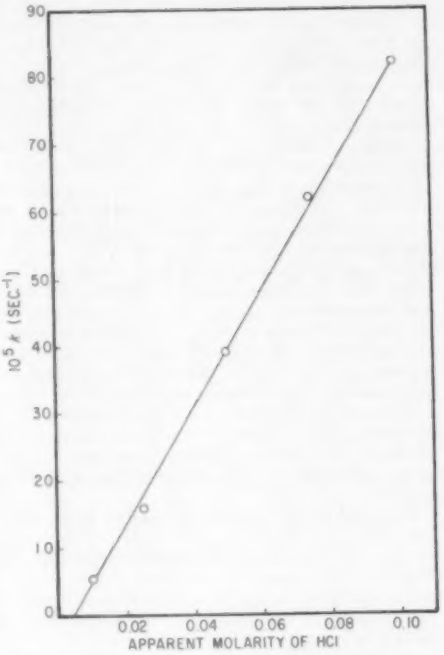

Fig. 2.—Rate constants, k in sec.$^{-1}$, for the pseudo first-order degradation of psicofuranine as a function of apparent HCl molarity at 40°.

tion is plotted against time. The catalytic effect of acid concentration is apparent from the increase in negative slope with the increase in hydrochloric acid molarity. The evaluated rate constants for the pertinent conditions used are given in Table I.

If the reaction is truly bimolecular with respect to acid and psicofuranine, the apparent first-order rate constant at constant hydrochloric acid concentration should not vary significantly with psicofuranine concentration. This point is confirmed by the consistency of the apparent first-order rate constants at 0.100 *M* HCl for three different initial concentrations of psicofuranine (see runs 5 through 10 in Table I). The dependence of the apparent first-order rate constants, k, on HCl concentration is graphically given in Fig. 2 according to Eq. 3. The apparent $[HCl]_A$ value when $k = 0$ is 0.004 which agrees well with the psicofuranine concentration at 1 mg./ml., i. e., $[P]_0 = 0.0037$. The slope of Fig. 2 is $kn^+ = 0.00374$ moles/L./min. The per cent purity of the psicofuranine by titration on assumption of the molecular formula (2) is 96.3%.

Correlation of the Chemical and Biological Assays of Psicofuranine.—In an attempt to correlate the chemical (10, 11) and biological (6) assays of psicofuranine, a kinetic study was made at 40° in 0.100 *M* HCl and aliquots removed and assayed by both methods. The aliquots for biological assay were immediately neutralized and submitted on the basis of 80 γ/ml. of the original material. The same psicofuranine as used in the study in the same slightly saline solutions was used to establish the standard curve for the plate-disk assay, i. e., zone diameter in mm. *vs.* log concentration. A discrep-

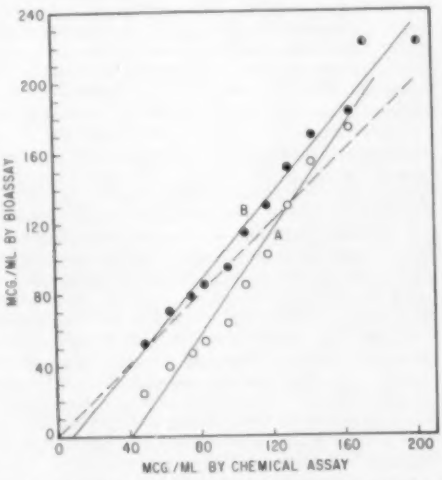

Fig. 3.—Correlation of disk-plate assay against *S. aureus* and the spectrophotometric measurement on the product from reaction of degraded (0.1 *N* HCl, 40°) psicofuranine with diphenylamine. Curve *A* is based on the standard curve for bioassay set up on psicofuranine alone. Curve *B* is based on the standard curve for bioassay predicted for psicofuranine with the amounts of adenine present in the aliquots to be assayed. The dashed line is theoretical.

ancy became immediately apparent. A plot of the potency by biological assay against purity by chemical assay showed an intercept not passing through the origin (see Fig. 3, curve A). Either this was an artifact of one of the assays or a certain fraction of the intact nucleoside had no biological activity.

This latter hypothesis and that of an artifact in the chemical assay appeared improbable since the acid degradations are elegantly pseudo first-order (see Fig. 1). Also, the chemical assay absorbance was experimentally shown to be a linear function of the concentration of the nucleoside.

A parallel experiment was again run (runs 9 and 10, Table I) whereby aliquots of the acid degrading psicofuranine were again removed, neutralized, and assayed by both procedures. One of these studies was as before. An initially equimolar amount of adenine, a hydrolytic product of the acid catalyzed psicofuranine degradations, was added to the other.

The standard curves for the psicofuranine plate-disk assay using *S. aureus* are given for these studies in Fig. 4 where curve A is the curve for psicofuranine alone and curve B is for psicofuranine with equimolar amounts of adenine.

The statistics for the regressions of bioassay on chemical assay are given in Table II. If the variation about regression is completely assigned to the bioassay, the estimated standard deviation of a single assay, S_y, is ± 10 γ/ml. for material assayed in the range 40–80 γ/ml., i. e., an estimated error of 12% in bioassay.

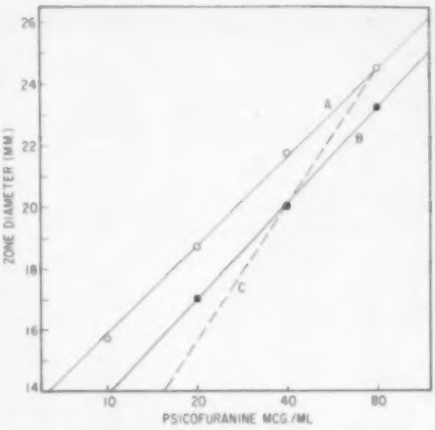

Fig. 4.—Standard curves for the disk-plate assay of psicofuranine using *S. aureus*, the zone size in mm. against the concentration in γ/ml. Curve *A* is for psicofuranine alone, curve *B* is for psicofuranine with a molar equivalent of adenine, the curve *C* is the constructed curve to account for the effect of adenine on zone size for a given antibiotic concentration when adenine is produced during acid catalyzed degradation.

TABLE II.—STATISTICS OF THE REGRESSION OF PSICOFURANINE CONTENT BY THE BIOLOGICAL ASSAY (y) AGAINST THE CHEMICAL ASSAY (x)[a]

	Biological Assay Based on Standard Curves	
	Psicofuranine Alone	Psicofuranine Corrected for Adenine Effects
Equation of regression, $y = mx + b$	$y = 1.46x - 61.2$	$y = 1.22x - 11.2$
Standard deviation of slope, S_m	0.090	0.066
Standard deviation of intercept, S_b	11.4	8.3
tS_m	0.202	0.15
tS_b	25.3	18.5
Standard deviation about regression, S_y	14.6	10.3
Number of items, n	12	12

[a] The psicofuranine aliquots were taken at various times on degradation in 0.100 *M* HCl at 40°.

DISCUSSION

The Apparent Discrepancy Between the Chemical and Biological Assays.—The bioassay of aliquots of a psicofuranine-0.1 *M* HCl solution maintained at 40° gave significantly less estimates of potency with time than the chemical assay which measured intact nucleoside (see curve A, Fig. 3). However, when adenine, a product of the hydrolysis, was added to the psicofuranine, a diminution of zone size for a comparable psicofuranine concentration was apparent in the standard curves for the plate-disk assay. Compare curves A and B in Fig. 4. If the point on the psicofuranine standard curve (curve A, Fig. 4) of 80 mcg./ml. is assumed as valid for no adenine present in the assay of psicofuranine and if the point on the equimolar psicofuranine-adenine standard curve (curve B, Fig. 4) of 40 mcg./ml. is as assumed as valid for the half-hydrolyzed psicofuranine, then curve C, Fig. 4, can be constructed which could be the standard curve for assay when the levels of adenine in the acid-degraded nucleoside are considered. This assumes, of course, logarithmic effect on the diminution of zone size by the addition of adenine. A complete exposition of effects will be published by Hanka (12).

When the constructed standard curve C, Fig. 4, is used rather than the standard curve A, Fig. 4, without adenine, the regression of bioassay on chemical assay becomes curve B, Fig. 3, and as per the statistics of regression, Table II, this regression passes through the origin within experimental error. The chemical assay can be concluded as a true measure of biologically active psicofuranine.

Confirmatory evidence was obtained from the paper chromatography of the acid-degraded psicofuranine at the point in time when the biological assay (based on an adenine-free standard curve) was zero and the chemical assay was *ca.* 30% of the original nucleoside content. Definite amounts of nonhydrolyzed psicofuranine in the magnitude expected were separated by this chromatography and coincided in position and color development with the psicofuranine controls (5).

SUMMARY

The kinetic investigations of the acid catalyzed degradations of psicofuranine prove that the hydrolysis is bimolecular in acid and nucleoside.

Correlation of bioassay and chemical assay for psicofuranine produced an estimated standard deviation in per cent of assay value of 12 per cent for the former which must be controlled for adenine content. The chemical assay is a valid measure of psicofuranine. Adenine reverses the biological activity of psicofuranine as assayed by the plate-disk method.

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A Pharmacologic Study of the Effects of Various Pharmaceutical Vehicles on the Action of Orally Administered Phenobarbital*

By MARVIN H. MALONE†, ROBERT D. GIBSON, and TOM S. MIYA‡

The ED_{50}-ED_{16}/LD_0 of phenobarbital sodium orally to rats 5 cc./Kg. was 126–190/240 mg./Kg. Constant dosage for this study was 200 mg./Kg. or equivalent. Normalcy, instead of righting reflex regained, was used to define termination of narcosis. Phenobarbital sodium was more efficient than phenobarbital when given in aqueous agar solution-suspensions. Increasing viscosity by increasing sucrose concentration of vehicle progressively lengthened induction time. There was more rapid absorption from hypotonic than hypertonic solutions of phenobarbital sodium; yet there was less rapid absorption when given 10 cc./Kg. than 5 cc./Kg. using an 80 per cent sucrose vehicle. A sex and species variation in response was noted. Calculations utilizing log transformation data were used to summarize most accurately results obtained.

THE PRESENT CONCEPT of an elixir is a clear, sweetened hydroalcoholic liquid intended for oral use which may or may not contain active medicinal agents. Considerable attention has been given to elixirs as vehicles for drugs but most of this research has been purely pharmaceutical in nature, and has had as its goal increased pharmaceutical elegance rather than increased therapeutic efficiency. However, Seeberg and Dille (1) have studied the comparative gastrointestinal absorption of barbital, barbital sodium, and barbital elixir in the cat. Blood levels of barbital were reported one-half hour after oral dosing, but no data as to comparative pharmacologic response were presented. Hazleton and Hellerman (2) have conducted a thorough investigation of the effects of sucrose, dextrose, glycerol, propylene glycol, and ethanol on the response of sodium pentobarbital and Metrazol administered orally to mice. Their work indicated that the pharmacodynamics of an elixir would be characteristic only for that specific elixir and dependent primarily upon the pharmacologic nature of the medicinal agent and its concentration, and secondarily, upon the physical and pharmacologic nature of the specific ingredients that constitute the elixir vehicle and their relative concentrations. Their results were obtained on mice not fasted prior to testing, and consecutive testing was practiced with a minimum period of only one week between experiments.

The majority of research on barbiturates has been done with those classified as ultrashort to

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The major portion of this article is taken from the Doctorial Thesis of Marvin H. Malone.
† Fellow of the American Foundation for Pharmaceutical Education, 1956–1958. Present address: College of Pharmacy, University of New Mexico, Albuquerque.
‡ Present address: Department of Pharmacology, Purdue University, Lafayette, Ind.

intermediate in duration of action. Phenobarbital, a long-acting barbiturate, is the most widely used barbiturate in this country and has a wide variety of uses. Most pharmacologic research has concerned itself with the effects of barbiturates after their parenteral administration, in spite of the fact that most are consumed orally. This investigation was undertaken to elucidate the pharmacodynamics of phenobarbital elixir U. S. P. XIV (3).

EXPERIMENTAL

General.—Nebraska stock white rats were raised in the animal quarters of this laboratory from Wistar strain rats. After sex-separation at the age of four to six weeks, they were permanently numbered and housed in large colony cages. Animal quarters were kept at a temperature of approximately 25°. Diet consisted of Purina laboratory chow checkers and water, both *ad libitum*. White rats termed Manor Farm stock are animals of the Wistar strain purchased from Manor Farms, Staatsburg, New York, and maintained for one month prior to test in the animal quarters of this laboratory under conditions identical to those described for Nebraska stock rats. All rats were tested at approximately thirty weeks of age. White mice used were purchased from J. C. Landis Co. of Hagerstown, Maryland, and were of Webster-Swiss stock. They were housed for one week prior to test in the animal quarters of this laboratory under conditions as described for Nebraska stock rats. Only mice within the weight range of 16–22 Gm. were used for experimentation.

Twenty-four hours prior to test, all food was removed from the cages and the cages were swept clear of food particles and feces. Both rats and mice were housed in cages with coarse wire screen floors that allowed passage of feces. During fasting, water was allowed *ad libitum*. During the test period each animal was kept in an individual container and water was withheld. Laboratory temperature was maintained at 25.5–27.8° during the tests, as animals anesthetized with phenobarbital are markedly poikilothermic. All animals with loss of righting reflex were turned every hour to prevent hypostatic pneumonia. Prior to dosing, all animals were randomized and weighed to the nearest gram. Technique of oral administration was that described by Holck, *et al.* (4), utilizing a Davol No. 8 feeding tube attached to a precision grade syringe. The length of feeding tube allowed to enter the rat from the apparatus was 120 mm. This length just passed into the cardiac portion of the stomach of male and female rats of this age and weight. Oral dosing of mice was accomplished with a precision grade syringe attached to a 20-gauge, one and one-half-inch needle, the point of which had been cut off, edges rounded inward, and polished. Dosage volume was 5 cc./Kg. for rats and 20 cc./Kg. for mice, unless otherwise stated.

Positive loss of righting reflex (LRR) was recorded when the animal lay quietly on its side and did not attempt to right itself after four consecutive hand turnings of the animal. Positive recovery of righting reflex (RRR) was noted when the animal maintained itself in an upright position and promptly resisted four consecutive attempts to place the animal first on one side and then on the other. Time of normalcy (N) was noted when four consecutive attempts by the investigator failed to place first the right and then the left hind leg of the rat in an extended position back of the animal. Furthermore, normalcy was not recorded unless the rat was grossly free of ataxia when placed on the floor free to roam. Time of death was recorded upon the cessation of respiration. After death all animals were autopsied. Having once been on test, the surviving rats and mice were not used for further experimentation. All weighings of drugs were done using an analytical balance. Solution-suspensions were prepared in volumetric flasks of at least 50-cc. capacity at 20°, and were administered within twenty-four hours after preparation.

RESULTS

Phenobarbital Sodium Dose-Response Curve.—Documentation of the oral dose-response curve of phenobarbital sodium was necessary as a point of departure for the following work. Results are summarized in Table I. Pooling the male and female data the oral narcotic ED_{50} as calculated by the log probit method (5) was 126 mg./Kg. with a standard error of ±11.5. Assuming a parallel lethal slope, the oral LD_{50} was estimated to be 360 mg./Kg. The oral therapeutic index (LD_{50}/ED_{50}) and safety index (LD_5/ED_{95}) were estimated to be 2.86 and 1.26, respectively. The 3.36% solution used to dose the 168.18 mg./Kg. dosage level was about isotonic. The slower induction time seen with the next higher dosage group could indicate less rapid absorption by the hypertonic 4% solution, in spite of the increase in dosage.

The common end point used to indicate termination of narcosis is RRR. However, for long-acting barbiturates such as phenobarbital where detoxification rates are slow and excretion rates equally so, this end point was not adequate. Animals receiving the higher doses of phenobarbital sodium were observed to have as many as five positive RRR end points. The borderline between sleep and nonsleep was so narrow that the animals were temporarily aroused by minor laboratory noises such as conversation, only to return to sleep once the disturbance had ceased. Once N was achieved, the rats did not relapse. Since the ED_{95} was calculated to be 190 mg./Kg., the constant equimolecular dosage adopted for all following work was 200 mg./Kg. for phenobarbital sodium and 182.7 mg./Kg. for phenobarbital. At this dosage level any potentiation or antagonism of the barbiturate response should be apparent.

Toxicity of Phenobarbital Elixir and Elixir Controls.—Rats were dosed with phenobarbital elixir U. S. P. XIV and appropriate elixir controls as shown in Table II. The elixir produced a very rapid nonphenobarbital-like induction time with death of respiratory arrest following within two hours after administration. Animals receiving only ethanol slept for an average of almost ten hours. Rats receiving the amaranth-glycerol-sucrose blank displayed a true loss of righting reflex, followed

TABLE I.—LOG DOSE SCREEN OF PHENOBARBITAL SODIUM ORALLY TO NEBRASKA STOCK RATS

Dosage, mg./Kg.	Sex	Rats Per Group	Mean Duration Time: Inj.-LRR, min.	LRR-N, hr.	Inj.-N, hr.	Inj.-Death hr.[a]
84.09	M	5	....	....	....	...
	F	5	....	....	....	...
100.00	M	5	....	....	....	...
	F	5	164(2)[b]	9.8(2)	12.6(2)	...
118.92	M	5	113(3)	11.4(3)	13.3(3)	...
	F	5	111(5)	11.3(5)	13.2(5)	...
141.42	M	5	69(2)	14.0(2)	15.1(2)	...
	F	5	104(3)	14.4(3)	16.1(3)	...
168.18	M	5	63(3)	21.8(3)	22.9(3)	...
	F	5	87(5)	21.2(5)	22.6(5)	...
200.00	M	5	76(5)	24.1(5)	25.3(5)	...
	F	5	137(5)	25.5(5)	27.7(5)	...
237.84	M	5	49(5)	≤40.7(5)[c]	≤41.5(5)	...
	F	5	54(5)	≤35.6(5)	≤36.5(5)	...
282.84	M	5	64(5)	≤40.9(4)	≤42.0(4)	2.2(1)
	F	5	44(5)	≤44.2(4)	≤45.0(4)	3.3(1)

[a] Death by respiratory arrest.
[b] Figures in parentheses represent the number of rats upon which the means is based.
[c] ≤ Designates: equal to or less than.

TABLE II.—PHENOBARBITAL ELIXIR AND VARIOUS ELIXIR CONTROLS ORALLY TO MALE NEBRASKA STOCK RATS[a]

Test Preparation	Rats Per Group	Mean Duration Time: Inj.-LRR, min.	LRR-N, hr.	LRR-Death, hr.[b]	Inj.-Death, hr.[b]	A. F.[c]	N. F.[d]
Phenobarbital elixir	10	13(10)[e]	...	1.5(10)	1.7(10)	10	9
15% Ethanol in water	7	7(7)	9.8(7)	....	....	..	..
Sweet orange peel tincture in water	7	...	...	....	....	..	..
Amaranth-glycerol-sucrose in water	7	53(7)	...	0.2(7)	1.1(7)	7	2
Amaranth-sucrose in water	7	...	...	....	....	..	..
45% Glycerol in water	7	55(7)	...	≥0.04(7)[g]	1.0(7)	5	2
90% Glycerol in water[f]	7	183(7)	...	≥0.07(7)	3.1(7)	6	1

[a] Phenobarbital dosage as the elixir was 182.7 mg./Kg. Dosage volume for the first six test preparations was 45.7 cc./Kg.
[b] Death by respiratory arrest. Death for all animals receiving glycerol was preceded by clonic convulsions.
[c] Number of rats with anal flow of test solution noted prior to death.
[d] Number of rats with nasal flow of test solution after death.
[e] Figures in parentheses represent the number of rats upon which the mean is based.
[f] Dosage volume was 22.8 cc./Kg.
[g] ≥ designates: equal to or greater than.

shortly by clonic convulsions and death of respiratory arrest. This activity appeared to be due solely to the presence of glycerol. Decreasing the dosage volume, yet administering an equivalent amount of glycerol produced essentially the same response although induction time was significantly more prolonged. Most animals receiving glycerol displayed a fluid diarrhea soon after dosage. In certain cases this anal discharge was bloody. Concurrent with muscular relaxation after death, fluid was observed flowing from the nostrils of some of these rats. Upon autopsy the lungs were normal in size and color, although distension of the stomach and intestines was noted.

Phenobarbital and Phenobarbital Sodium in Aqueous Vehicles.—Male and female rats were orally dosed with either phenobarbital sodium in an aqueous solution, phenobarbital sodium in 0.25% aqueous agar, or phenobarbital finely suspended in 0.25% aqueous agar as shown in Table III, part 1.

Attempts to apply statistics to the raw data revealed that the variances of the groups receiving phenobarbital sodium were not comparable to the variance of the group receiving phenobarbital. Conversion of the raw data into natural log transformation data revealed upon statistical treatment with the F test that variances between the groups were then comparable and that the t test could be validly applied to this data. This finding further indicated that group mean and standard error of the mean determinations should be calculated using log transformation data rather than raw data in order to depict most accurately the research results of this experiment. A group mean calculated from log transformation data is termed a geometric mean. The 95% confidence limits for the geometric mean were calculated from the log transformation data and are two times the standard error of the mean. Statistical calculations followed the format outlined by Bliss and Calhoun (6). Results are summarized in Table III, part 2.

The induction time of rats dosed with phenobarbital was approximately three and one-half times longer than that recorded for the other two groups receiving the salt. Differences in vehicles did not grossly affect induction time for phenobarbital sodium. The pH of the 4% aqueous solution of phenobarbital sodium was 9.1, while that of the 0.25%

TABLE III, PART 1.—PHENOBARBITAL AND PHENOBARBITAL SODIUM ORALLY IN AQUEOUS VEHICLES TO MANOR FARM RATS[a]

Test Preparation	Test Group Code	Sex	Geometric Mean Duration Time: Inj.-LRR, min.	LRR-N, hr.	Inj.-Death, hr.[b]
Phenobarbital sodium in distilled water	PNa.W.M	M	27 (10)[c] 23–32[d]	28.9 (9) 25.7–32.6	4.5 (1)
	PNa.W.F	F	31 (10) 25–39	34.3 (8) 32.6–36.2	4.5 (2)
Phenobarbital sodium in 0.25% agar	PNa.A.M	M	30 (10) 24–36	28.5 (10) 26.5–30.6	...
	PNa.A.F	F	39 (10) 34–46	34.0 (8) 31.5–36.6	5.0 (2)
Phenobarbital in 0.25% agar	P.A.M	M	116 (10) 92–145	27.5 (10) 25.0–30.2	...
	P.A.F	F	126 (10) 94–170	33.4 (10) 31.8–35.0	...

[a] Dosage used was 200.0 mg./Kg. for phenobarbital sodium and 182.7 mg./Kg. for phenobarbital. Ten rats were dosed per sex per group.
[b] Death by respiratory arrest.
[c] Figures in parentheses represent the number of rats upon which the mean is based.
[d] Range figures indicate the 95% confidence limits of the geometric mean. These figures are calculated using log transformation data.

TABLE III, PART 2.—TESTS OF SIGNIFICANCE

Comparison[a]	t Test, Observed P: Inj.-LRR	LRR-N
PNa.W.M *vs.* PNa.A.M	>0.50	>0.50
PNa.W.M *vs.* P.A.M	<0.001	>0.50
PNa.W.F *vs.* PNa.A.F	0.10–0.05	>0.50
PNa.W.F *vs.* P.A.F	<0.001	0.50–0.25
PNa.W.M *vs.* PNa.W.F	0.50–0.25	0.025–0.01
PNa.A.M *vs.* PNa.A.F	0.05–0.025	0.005–0.001
P.A.M *vs.* P.A.F	>0.50	0.005–0.001
PNa.W.M and PNa.A.M *vs.* P.A.M	<0.001	0.50–0.25
PNa.W.F and PNa.A.F *vs.* P.A.F	<0.001	>0.50
PNa.W.M and PNa.A.M *vs.* PNa.W.F and PNa.A.F	0.05–0.025	<0.001

[a] See Table III, part 1 for test group code names.

agar solution was 8.8. The 3.65% solution-suspension of phenobarbital in 0.25% agar had a pH of 7.2. The slower onset noted with phenobarbital may be due to a difference in pH. Brodie and co-workers (7) postulated that acidic drugs are rapidly absorbed in their lipid soluble nonionized state from the highly acid stomach and are not well absorbed in their lipid insoluble ionized state from the alkaline intestine. Discrepancies in this concept were explained by invoking the Meyer-Overton partition coefficient (8, 9). The gastrointestinal and blood-brain barriers are considered to be primarily lipid in nature. According to the Brodie concept the alkaline preparations of phenobarbital sodium should be poorly absorbed from the stomach, while the neutral solution-suspension of phenobarbital should be well absorbed. Passage of all three preparations into the alkaline intestine should hinder absorption. As a result of this, the animals dosed with the neutral solution-suspension should have a much shorter onset of narcosis. Using intact laboratory animals in a standardized clinical test situation, this study clearly indicated the opposite situation. Rate of solution for phenobarbital in the gastrointestinal contents may explain the results observed here. Table III, part 2, shows that there is a highly significant sex difference in regard to duration of narcosis, the females sleeping about five hours longer. The pooled data of the two groups receiving phenobarbital sodium indicated that there was also a significant sex difference as to onset of LRR, with the males showing a shorter induction time.

Phenobarbital Sodium to Mice.—In order to determine whether the sex difference previously unreported for long acting phenobarbital would be observed in another species, mice were dosed orally with 200 mg./Kg. of phenobarbital sodium using a 1% aqueous solution. Results are summarized in Table IV. Females had a significantly faster induction time (P 0.05–0.025) and a greater mortality from respiratory arrest as compared with the males. There was no significance (P >0.50) between the sexes as to duration of lost reflex observed with survivors. This indicated a species variation in response.

TABLE IV.—200 MG./KG. PHENOBARBITAL SODIUM ORALLY TO MICE

Sex	Mice Per Group	Geometric Mean Duration Time: Inj.-LRR, min.	LRR-RRR, hr.	Inj.-Death, hr.
M	21	25 (21)[a] 19–34	18.3 (19) 13.7–24.5	31.2 (2)
F	21	18 (21) 16–20	17.8 (15) 14.0–22.5	35.1 (6)

[a] Figures in parentheses represent the number of mice upon which the mean is based, while the range figures indicate the 95% confidence limits for the geometric mean.

Phenobarbital Sodium in Various Sucrose Syrups.—A 10% sucrose solution is considered to be isotonic. To test the effect of hypotonic, isotonic, and hypertonic sucrose solutions upon the response

to phenobarbital sodium, 10 animals per sex, per test treatment were dosed orally as shown in Table V, part 1. Statistical evaluation is summarized in Table V, part 2.

With increasing concentration of sucrose in the vehicle there was a progressive lengthening of induction time. Also, there was a highly significant sex variation in regard to duration of narcosis, with the females sleeping up to eight hours longer than the males. As viscosity and surface tension may be the factors modifying the response in this experiment, these physical properties were measured using the Du Noüy tensiometer and the Ostwald pipet, and are correlated with induction time in Fig. 1. Each point in Fig. 1 represents the mean of five determinations made at 37°. Qualitatively the viscosity and induction time curves were similar, while the surface tension curve was qualitatively different. This indicated that viscosity was the major contributing factor for the observed lengthening of induction time. In this instance viscosity parallels osmotic pressure.

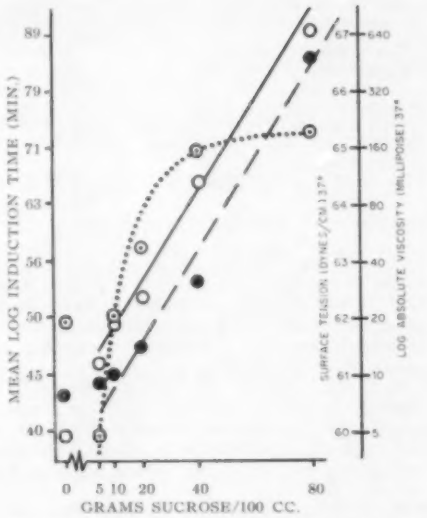

Fig. 1.—Relationship of induction time after 200 mg./Kg. phenobarbital sodium to the viscosity and surface tension of various sucrose syrup vehicles: ○, solid line, induction time; ●, dash line, viscosity; ⊙, dotted line, surface tension.

TABLE V, PART 1.—200 MG./KG. PHENOBARBITAL SODIUM ORALLY IN VARIOUS SUCROSE SYRUPS TO NEBARSKA STOCK RATS[a]

Test Vehicle	Sex	Geometric Mean Duration: Inj.-LRR, min.	Geometric Mean Duration: LRR-N, hr.
Water	M	42 (32–54)[b]	19.9 (15.9–24.9)
	F	37 (32–44)	26.3 (24.5–28.3)
5% Sucrose	M	43 (37–51)	22.0 (18.8–25.8)
	F	48 (39–60)	22.6 (19.8–25.8)
10% Sucrose	M	45 (40–51)	20.0 (17.8–22.3)
	F	54 (46–63)	24.9 (21.8–28.4)
20% Sucrose	M	48 (41–55)	21.3 (18.1–24.9)
	F	57 (43–75)	24.5 (22.2–27.0)
40% Sucrose	M	57 (39–82)	16.5 (10.3–26.3)
	F	77 (54–111)	24.5 (19.4–31.0)
80% Sucrose	M	94 (67–133)	18.9 (14.9–23.9)
	F	86 (63–118)	24.2 (20.6–28.4)

[a] Ten rats were dosed per sex per test treatment.
[b] Figures in parentheses indicate the 95% confidence limits of the geometric mean.

Phenobarbital Sodium at Two Dosage Volumes in Water and 80% Sucrose Syrup.—Female rats were dosed as shown in Table VI, part 1. Statistical evaluation of the data is summarized in Table VI, part 2. When 200 mg./Kg. phenobarbital sodium was administered in aqueous solution it was more rapidly effective from a dilute (2%) rather than from

TABLE V, PART 2.—TESTS OF SIGNIFICANCE

Comparison:	t Test, Observed P			
	Inj.-LRR		LRR-N	
Water vs.	Between Vehicles	Between Sexes	Between Vehicles	Between Sexes
5% Sucrose	0.10–0.05	>0.50	>0.50	0.05–0.025
10% Sucrose	0.025–0.01	>0.50	>0.50	<0.001
20% Sucrose	0.025–0.01	>0.50	>0.50	<0.001
40% Sucrose	0.005–0.001	>0.50	0.50–0.25	0.025–0.01
80% Sucrose	<0.001	0.50–0.25	0.50–0.25	<0.001

TABLE VI, PART 1.—EFFECTS OF DIFFERENT DOSAGE VOLUMES ON RESPONSE OF 200 MG./KG. PHENOBARBITAL SODIUM ORALLY TO FEMALE NEBRASKA STOCK RATS[a]

Dosage Volume cc./Kg.	Vehicle	Test Group Code	Geometric Mean Duration Time: Inj.-LRR, min.	Geometric Mean Duration Time: LRR-N, hr.	Inj.-Death,[b] hr.
5.0	Water	PNa.W.5	49 (10)[c]	27.3 (9)	2.8 (1)
			32–74[d]	26.2–28.5	
10.0	Water	PNa.W.10	29 (10)	28.5 (7)	5.7 (3)
			24–35	27.8–29.3	
5.0	80% Sucrose	PNa.S.5	80 (10)	23.2 (10)	....
			60–106	17.8–30.3	
10.0	80% Sucrose	PNa.S.10	129 (10)	23.1 (9)	6.1 (1)
			106–157	20.7–25.7	

[a] Ten rats were dosed per test treatment.
[b] Death by respiratory arrest.
[c] Figures in parentheses represent the number of rats upon which the mean is based.
[d] Range figures indicate the 95% confidence limits of the geometric mean.

TABLE VI, PART 2.—TESTS OF SIGNIFICANCE

Comparison	t Test, Observed P	
	Inj.-LRR	LRR-N
PNa.W.5 *vs.* PNa.W.10	0.05–0.025	0.50–0.25
PNa.S.5 *vs.* PNa.S.10	0.025–0.01	>0.50
PNa.W.5 *vs.* PNa.S.5	0.10–0.05	0.50–0.25
PNa.W.10 *vs.* PNa.S.10	<0.001	0.01–0.005

a concentrated (4%) solution, as shown by a significantly shorter induction time and a 30% incidence of fatalities. However, when large concentrations of sucrose in the vehicle rendered phenobarbital's contribution to osmotic pressure insignificant, the effects of administering a more dilute solution of phenobarbital sodium was actually reversed, as shown by a prolonged induction time and a significantly reduced duration of narcosis. This indicated that while the osmotic pressure of the vehicle can modify response, the dosage volume is also an important factor.

Phenobarbital in Ethanol-Glycerol-Water Mixtures.—From the data of Krause and Cross (10) various combinations of ethanol and glycerol were selected in which 182.7 mg./Kg. of phenobarbital could be dosed at 5 cc./Kg. With only four rats dosed per test treatment, only qualitative conclusions could be drawn which corroborate the findings of Hazelton and Hellerman (2).

DISCUSSION

Phenobarbital elixir U. S. P. XIV contains three ingredients with pharmacologic activity: phenobarbital, ethanol, and glycerol. It was impossible to determine the relative merit of the intact elixir because the narcotic effect of phenobarbital was both qualitatively and quantitatively changed by the various actions of ethanol and glycerol. Pharmacologically inactive sucrose was shown to quantitatively alter the phenobarbital response. A proposed new elixir can be formulated on a pharmacologic basis. It should be a dilute hydroalcoholic solution of phenobarbital sodium. The presence of ethanol would lend a certain amount of pharmaceutical stability as well as supplementing and hastening the action of phenobarbital sodium. A synthetic sweetener, such as cyclamate sodium N. N. D. which does not appreciably change viscosity, should be substituted for the sucrose and glycerol. Further flavoring is indicated. This postulated elixir fulfills the classic definition, but would avoid the undesirable vehicle pharmacodynamics found in phenobarbital elixir U. S. P. XIV by this study and others.

SUMMARY

1. Using a constant dosage volume of 5 cc./Kg. the oral narcotic ED_{50} for phenobarbital sodium administered to rats was found to be 126 mg./Kg. (± 11.5). The dosage level adopted for this study was 200 mg./Kg. or its equivalent, since the ED_{95} was calculated to be 190 mg./Kg. The safety index (LD_5/ED_{95}) was estimated to be equal to 1.26.

2. The pharmacologic end point of righting reflex regained (RRR) cannot be used to describe accurately the action of long acting barbiturates. A new end point, normalcy, was defined.

3. Ethanol and glycerol were shown to modify qualitatively and quantitatively the effect of the phenobarbital in phenobarbital elixir U. S. P. XIV.

4. Group mean and standard error of the mean calculations for induction time and duration of barbiturate narcosis must be calculated from common log transformation data to describe experimental results most accurately.

5. Phenobarbital sodium was more efficient in inducing narcosis than phenobarbital, when these compounds were administered in aqueous solution-suspensions of low viscosity.

6. Increasing concentrations of sucrose in aqueous test solutions of phenobarbital sodium progressively lengthened the induction time for narcosis. This appeared to be a function of viscosity rather than surface tension.

7. Phenobarbital sodium was more rapidly effective in dilute aqueous solutions than in concentrated aqueous solutions.

8. An equal dosage of phenobarbital sodium was less rapidly effective when administered 10 cc./Kg. than 5 cc./Kg. in an 80% aqueous solution of sucrose.

9. There was found a definite sex variation in response to phenobarbital and phenobarbital sodium administered orally to rats of the Wistar strain. Males had a shorter duration of narcosis as well as a somewhat shorter induction time as compared to the females. No colony variation was seen. A qualitative species variation was observed between rats and mice.

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Substituted Xanthines II*

Preparation and Properties of Some (2-Hydroxyalkyl)trimethylammonium Theophyllinates

By FREDERICK VIDAL† and THEODORE I. FAND‡

A new and convenient method has been developed for the large scale preparation of (2-hydroxyethyl)trimethylammonium theophyllinate.[1] The synthesis of several (2-hydroxyalkyl)trimethylammonium theophyllinates was accomplished by the addition of trimethylamine to various epoxyalkanes and then reacting the resulting intermediate with theophylline or 8-substituted theophylline derivatives. Various properties of these compounds are described.

THE METHYL derivatives of xanthine, particularly theophylline, have been used for many years as therapeutic agents in bronchial asthma, angina pectoris, and as mild diuretics. Theophylline has also found clinical application for myocardial stimulation and has been employed occasionally for the treatment of hypertension.

Although the therapeutic value of this drug is well founded, it is not without undesirable side effects. Of these, the most important is that it causes gastric irritation with concomitant nausea and vomiting. Another disadvantage of theophylline is its very limited solubility in water. In order to increase its therapeutic effectiveness, particularly in oral administration, preparations with enhanced water solubility were investigated. Certain double compounds of theophylline with salts and aliphatic amines showed considerably higher solubility. Several such addition products or mixtures have been reported, including aminophylline, theophylline-methylglucamine, and theophylline sodium glycinate (1, 2).

In contrast to the double compounds mentioned above, it was shown that the condensation of choline with theophylline afforded an ionic compound which possessed properties of a true salt (3).

(2-Hydroxyethyl)trimethylammonium theophyllinate was previously prepared by two methods. The first (4) consisted of reacting choline base with an alcoholic suspension of theophylline. The second method (3) involved heating theophylline with an excess of choline bicarbonate in an aqueous medium until the evolution of carbon dioxide ceased. The reaction product was then obtained upon concentrating the solution under reduced pressure. While these procedures were suitable for laboratory work, they were not satisfactory for large scale operation.

The method of choice (5) for preparing this quaternary ammonium salt was patterned after Wurtz's well-known synthesis (6) of choline with several pertinent modifications. Good yields of several (2-hydroxyalkyl)trimethylammonium theophyllinates, free of inorganic salts, resulted from the reaction between trimethylamine, various epoxyalkanes, and theophylline or its 8-substituted derivatives.

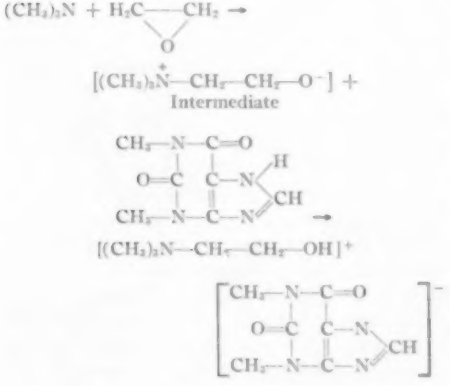
(2 - Hydroxyethyl)trimethylammonium theophyllinate was conveniently formed in high yields when ethylene oxide was used in this reaction. In order to determine the optimal reaction conditions, the effects of the following variables were studied: (*a*) pressure, (*b*) solvents, (*c*) temperature, and (*d*) presence of water. It was found that the yields for (2-hydroxyethyl)trimethylammonium theophyllinate ranged between 83 and 97% of the theoretical under the different conditions employed. The best yield could be obtained by the use of anhydrous isopropanol as solvent and by cooling the starting materials at the beginning of the reaction to −10°. Furthermore, it was possible to carry out the reaction at atmospheric pressure if these conditions were maintained. The reaction was found to be vigorous and exothermic both under completely anhydrous conditions or in the presence of catalytic amounts of water. (2-Hydroxyethyl)trimethylammonium theophyllinate was the sole reaction product in all but one of these experiments. In the latter case, 7-(2-hydroxyethyl)-theophylline was isolated as a minor component in 5% yield, while the major product was the quaternary ammonium salt, obtained in 89% yield. Table I summarizes most of the results obtained in the investigation of the optimal reaction conditions for the preparation of (2-hydroxyethyl)trimethylammonium theophyllinate or its 8-substituted derivatives.

* Received August 31, 1959, from the Research Laboratories, Nepera Chemical Co., Inc., Yonkers 2, N. Y.
For the preceding paper in this series see Reference 3.
† Present address: Wallace and Tiernan, Inc., Belleville, N. J.
‡ Present address: Warner-Lambert Research Institute, Morris Plains, N. J.
[1] Choledyl is the registered trade mark of Warner-Lambert Pharmaceutical Co., for its brand of choline theophyllinate.

If the 8-bromo-, chloro-, or nitro-theophylline derivatives were employed, the sole reaction product isolated in each instance, was (2-hydroxyethyl)trimethylammonium 8-bromotheophyllinate, (2-hydroxyethyl)trimethylammonium 8-chlorotheophyllinate, and (2-hydroxethyl)trimethylammonium 8-nitrotheophyllinate, respectively.

The reaction of theophylline with propylene oxide and trimethylamine led to the formation of only one product, namely, (2-hydroxypropyl)-trimethylammonium theophyllinate, obtained in a yield of 67%. However, when 1,2-epoxybutane was used instead of 1,2-epoxypropane, two products were isolated; namely, (2-hydroxybutyl)trimethylammonium theophyllinate (in 48% yield) and 7-(2-hydroxybutyl)-theophylline (in 26% yield).

On the basis of our findings, the formation of (2-hydroxyethyl)trimethylammonium theophyllinate is postulated to proceed as follows: in the initial step trimethylamine reacts with the reactive ethylene oxide by opening the oxirane ring. The resultant intermediate then reacts with theophylline to give the final product. A possible mechanism for the reaction, under anhydrous conditions, may be represented schematically as follows:



In the presence of water, however, it is assumed that the intermediate first formed is choline, which then condenses with theophylline, yielding the desired quaternary ammonium salt.

Several attempts were made to prepare (1-methyl - 2 - hydroxypropyl)trimethylammonium theophyllinate and (2-hydroxyphenethyl)trimethylammonium theophyllinate by heating theophylline and trimethylamine with 2,3-epoxybutane and with styrene oxide, respectively. We were unable, however, to obtain the desired quaternary ammonium salts in crystalline form by the described method. In these instances, the only reaction products isolated and identified were the 7-(1-methyl-2-hydroxypropyl)- and 7-(2-hydroxyphenethyl)- derivatives of theophylline, respectively.

According to our results the yield of the corresponding quaternary ammonium salt decreased as the chain-length of the alkyl group of the epoxide increased, or if an aryl substituted epoxide was used. Simultaneously with the decrease in yields of the quaternary ammonium compounds, there was an increase in the amounts of the 7-(2-hydroxyalkyl)-theophyllines. Obviously, the formation of the latter theophylline derivatives competes with the main reaction and the proportion of these two different types of compounds obtained, depends on the alkyl chain length of the epoxide used.

A future publication will deal with the properties and a convenient procedure for the preparation of some 7-(2-hydroxyalkyl)-theophyllines.

EXPERIMENTAL[1]

Materials.—8-Bromotheophylline was prepared by the method of Biltz and Strufe (7) and 8-chlorotheophylline was prepared according to Fischer and Ach (8). 8-Nitrotheophylline was obtained according to a previously described method (3).

Trimethylamine and the different epoxides were obtained from commercial sources.

Procedure.—The general reaction procedure for the preparation of the compounds listed in Table I is illustrated by the following examples. In the experiments in which water was used as the solvent, it was necessary to evaporate the solution *in vacuo* to about one-third of its original volume in order to isolate the reaction products. Isopropanol was then added until a precipitate formed upon cooling.

(2-Hydroxyethyl)trimethylammonium Theophyllinate.—A solution of 28 Gm. (0.47 mole) of dry trimethylamine in 100 ml. of anhydrous isopropanol was placed in the reaction flask and cooled to −10°. Twenty-one grams (0.48 mole) of ethylene oxide was added while stirring, followed by 65 Gm. (0.36 mole) of anhydrous theophylline. During this period the temperature was maintained around −10°. The slurry formed was stirred vigorously at −10° for one hour and at room temperature for another hour. During this latter time, an exothermic reaction occurred and the temperature of the mixture rose to 65°. When the reaction subsided, the slurry was stirred at about 60° for an additional one and one-half hours. Upon cooling in ice, the product was filtered on a Büchner funnel and washed thoroughly with cold isopropanol. The yield of (2-hydroxyethyl)trimethylammonium theophyllinate was 99 Gm. (97%). The product melted at 186–187° and did not depress the melting point of an authentic sample of (2-hydroxyethyl)tri-

[1] All melting points have been corrected and were taken in capillaries.

TABLE I.—REACTIONS OF ETHYLENE OXIDE WITH TRIMETHYLAMINE AND THEOPHYLLINE OR 8-SUBSTITUTED THEOPHYLLINE DERIVATIVES

No.	Trimethylamine	Purine Derivative	Solvent, ml.	Pressure	Reaction: Time, hr.	Reaction: Temp. °C.	Products Name	M. P., °C.	Yield, %
1	25% Aqueous solution	Th[a]	100 H_2O	Sealed tube	48	20	ChTh[b]	188–188.5[c, f]	83.5
2	25% Aqueous solution	Th[e]	100 H_2O	Atmospheric	1 48	100 20	ChTh[b]	187.5–188.5[c, f]	85
3	Anhydrous	Th[d]	220 Isopr. + 20 H_2O	Atmospheric	1 2	100 0	ChTh[e]	187.5–188.5[c, f]	89.5
4	25% Aqueous solution	BrTh[g]	50 H_2O	Sealed tube	2 48	60 20	ChBrTh	65–65.5[f, h]	85.5
5	25% Aqueous solution	ClTh[g]	50 H_2O	Atmospheric	1 48	100 20	ChClTh	97–99[f, i]	86
6	Anhydrous	NTh[g]	180 Isopr. + 20 H_2O	Atmospheric	1 2 2	50 0 60	ChNTh	248[f, j]	87.5

Th = theophylline; BrTh = 8-bromotheophylline; ClTh = 8-chlorotheophylline; NTh = 8-nitrotheophylline; Isopr. = isopropanol; ChTh = (2-hydroxyethyl)trimethylammonium theophyllinate; ChBrTh = (2-hydroxyethyl)trimethylammonium 8-bromotheophyllinate; ChClTh = (2-hydroxyethyl)trimethylammonium 8-chlorotheophyllinate; ChNTh = (2-hydroxyethyl)trimethylammonium 8-nitrotheophyllinate. [a] A 1:1:1 mole ratio of Th, base, and epoxide was used. [b] Unreacted Th, 7%, was also recovered. [c] Reported (3) m. p. 186°. [d] A 0.9:1:1 mole ratio of Th, base, and epoxide was used. [e] In addition to the quaternary ammonium compound, 4.6% of 7-(2-hydroxyethyl)-theophylline, m. p. 162–164° was also obtained in this experiment; this compound gave no melting point depression when admixed with an authentic sample of the same product. [f] Recrystallized from isopropanol. [g] A 0.75:1:1 mole ratio of 8-substituted Th, base, and epoxide was used. [h] Reported (3) m. p. 67°. [i] Dried at 60°. [j] Reported (3) m. p. 248°.

methylammonium theophyllinate, prepared by another method.

(2-Hydroxypropyl)trimethylammonium Theophyllinate.—In a three-necked flask equipped with a stirrer, gas inlet tube, and an addition funnel was placed 100 ml. of isopropanol. The flask was immersed in an ice bath, 31 Gm. (0.52 mole) of trimethylamine bubbled into the solvent, and 30 Gm. (0.52 mole) of propylene oxide added. To this solution, 72 Gm. (0.4 mole) of anhydrous theophylline was added slowly while stirring over a period of thirty minutes. The slurry which formed was then stirred vigorously at 5° for one hour and at room temperature for one and one-half hours, when the flask was equipped with a condenser in place of the inlet tube. During this latter time, an exothermic reaction took place and the temperature rose to about 60°. Finally, the reaction mixture was stirred at about 60° for an additional one and one-half hours. The slurry was cooled to 0°, the solid was filtered off and washed thoroughly with cold isopropanol. Recrystallization of the white product from isopropanol gave 80.3 Gm. (67.5%) of (2-hydroxypropyl)trimethylammonium theophyllinate, m. p. 186.5–187.5°. A mixed melting point with a sample of (2-hydroxyethyl)trimethylammonium theophyllinate showed a marked depression (m. p. 170–172.5°). (2-Hydroxypropyl)trimethylammonium theophyllinate was found to be very soluble in water (1:1) and a 1% aqueous solution had a pH of about 9.5.

Anal.[3]—Calcd. for $C_{13}H_{22}N_4O_3$: C, 52.5; H, 7.79; N, 23.56; neut. equiv., 297. Found: C, 52.95; H, 7.6; N, 24.0; neut. equiv., 294.

(2-Hydroxybutyl)trimethylammonium Theophyllinate.—To 50 ml. of isopropanol, cooled to 0°, was added 16 Gm. (0.27 mole) of trimethylamine and 18 Gm. (0.25 mole) of 1,2-butylene oxide while the temperature was maintained at 0°. This solution was stirred and 36 Gm. (0.2 mole) of anhydrous theophylline was introduced in small portions. After the final addition, the slurry which formed was stirred vigorously at 0° for one hour and at room temperature for an additional two hours. After the first hour, an exothermic reaction ensued and the mixture reached a temperature of 40°. When the reaction had subsided, the mixture was stirred at 60° for another hour. During this time, the suspended material originally present had dissolved. Upon cooling to 5°, the precipitate which formed was collected on a Büchner funnel and washed thoroughly with cold isopropanol, m. p. 150–154°. Two recrystallizations of the crude material from isopropanol gave 30.2 Gm. (48.5%) of (2-hydroxybutyl)-trimethylammonium theophyllinate, m. p. 164–166°. This salt was somewhat hygroscopic, very soluble in water, and a 1% aqueous solution gave an alkaline reaction (pH 9.5).

Anal.—Calcd. for $C_{14}H_{24}N_4O_3$: N, 22.5. Found: N, 22.9.

After concentrating the combined mother liquors to a small volume and subsequent cooling, 24 Gm. of a white crystalline precipitate, m. p. 132–137°, separated. This fraction was twice recrystallized from isopropanol to yield 13 Gm. (25.8%) of 7-(2-hydroxybutyl)-theophylline, m. p. 141–142.5°.

Anal.—Calcd. for $C_{11}H_{16}N_4O_3$: N, 22.22. Found: N, 22.6.

[3] Microanalyses for C and H were performed by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.; N analyses were carried out by Miss Ruth Becker; the neutral equivalent determination was made by Mr. Milton Geller.

SUMMARY

1. A series of (2-hydroxyalkyl)trimethylammonium theophyllinates has been prepared for evaluation of diuretic and antispasmodic activities.

2. A new and more convenient procedure for the synthesis of these compounds has been described.

3. The following new compounds have been characterized: (2 - hydroxypropyl)trimethylammonium theophyllinate, (2-hydroxybutyl)-trimethylammonium theophyllinate, and 7-(2-hydroxybutyl)-theophylline.

4. The compounds tested showed comparatively low toxicity. Previous research has disclosed effective bronchodilator action of some of these compounds.

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A Comparison of the Effects of Colchicine and Some Purified Veratrum Alkaloids on Nuclear Division in Roots of *Allium cepa* L.*

By DOUGLAS L. SMITH† and L. DAVID HINER

Purified alkaloidal mixtures and crystalline veratrum alkaloids were compared with colchicine as to effects on nuclear and cellular division. Chromosomal aberrations often resulted but appeared to be due to a different type of action.

THE REMARKABLE EFFECTIVENESS of colchicine to alter the nuclear mitotic processes in meristematic tissues is well established (1). Living cells respond almost universally to colchicine after a basic pattern which constitutes the colchicine mitosis (c-mitosis), in which selective inhibition of spindle fiber formation interferes with nuclear division at metaphase.

Many chemical agents other than colchicine have been used to produce nuclear and cytoplasmic changes in plants (2–9). In 1944, Witkus and Berger reported on "veratrine, a new polyploidy inducing agent" (10). According to these authors the cytological effects of this agent were similar to those of colchicine with a few differences in the mechanism of action by which polyploidy was produced. Because of the renewed interest in the active principles of veratrum and related genera (Zygadenus) by the medical profession within recent years, numerous investigators have attempted to isolate new veratrum-like extracts (11–13), to characterize the isolated derivatives (14–16), and to evaluate what, if any pharmacological activity resides in the agents thus obtained (17–19). With this renewed interest in *Veratrum viride* and related derivatives, many purified alkaloids heretofore unobtainable have now become available. Because of the close structural similarity between the alkaloids in veratrine and the alkaloids found in *Veratrum viride* Ait. and *Veratrum album* L., it was thought important to conduct a study to determine (*a*) whether some purified alkaloidal mixtures (Veriloid[1]) or crystalline alkaloids of veratrum (protoveratrine A[2]) possess the ability to modify the cycle of nuclear and cellular division, and (*b*) to determine how such activity compares in kind and degree to that already established for colchicine. The effects of colchicine on nuclear

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† Currently serving on active duty as instructor, Department of Chemistry, United States Air Force Academy, Colorado.
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[1] Veriloid, a mixture of active principles from *Veratrum viride* Ait. standardized for hypotensive activity to reference standard alkavervir, supplied by Dr. J. E. Campion, Riker Laboratories Inc., Los Angeles, Calif.
[2] Protoveratrine A, a purified alkaloidal fraction obtained from *Veratrum album* L., supplied by Dr. J. E. Campion, Riker Laboratories Inc., Los Angeles, Calif., and Dr. L. C. Weaver, Pitman-Moore Company, Indianapolis, Ind.

mitotic activity were investigated because this agent has become the agent of choice for experimental alteration of cell division processes and because it was desired to establish a comparison standard for subsequent studies on Veriloid and protoveratrine A. The results of this investigation provide the basis for this report.

METHODS

Onion bulbs, *Allium cepa* L., var. White Portugal, were used as the experimental plant. A solution of 2% 2-chloroethanol[1] in tap water was used to induce sprouting of new roots on the bulbs. The purpose of soaking the bulbs in the 2-chloroethanol solution was to induce the sprouting of more roots than would result from the use of tap water alone (20). After the roots had sprouted, constant solution temperatures at 30 ± 0.5° were maintained at all times. Root tips were prepared for microscopic examination by standard fixing, staining, and mounting procedures (21–24).

All intact cells observed in three scannings through the upper, middle, and lower third of a slide were recorded and the per cent of cells in any stage of nuclear mitotic division was calculated. Statistical analyses of the experimental data were performed by utilization of the analysis of variance technique after minor transformation of the original data to angles (25, 26).

For both colchicine and Veriloid, a population sample of 100 allium bulbs was randomly divided into 5 main groups of 20 bulbs each. Each of the main groups was further randomized and subdivided into 4 subgroups of 5 bulbs each. The 5 main groups consisted of 4 concentrations of the drug from 0.1 to 0.8% (concentrations represented colchicine U. S. P. or Veriloid equivalent to reference standard alkavervir). The remaining main group served as the control. The subgroups represented four different times of exposure to the requisite test solution, i.e., three, six, nine, or twelve hours.

In order to dissolve the Veriloid, a minimum amount of diluted acetic acid N. F. (1.4 ml./100 ml. of solution) was employed. This amount of acid (equivalent to 0.084 Gm. of $C_2H_4O_2$) was found by examination of the cells to be noninjurious to the root tissues. Special glass holders for the bulbs and alkaloidal solutions were constructed from Pyrex glassware (Fig. 1) which would hold approximately 2 to 4 ml. of solution. Each glass holder consisted of a watch glass to which a piece of glass tubing, sealed at one end, was heat-fused with simultaneous vacuum suction. The holders were suspended on a wire rack in the constant temperature bath.

Fig. 1.—Glass solution holder.

[1] Supplied by Distillation Products Industries, Rochester 3, N. Y.

In investigating the effects of protoveratrine A, a population sample of 25 allium bulbs was used and randomized into 5 groups of 5 bulbs each. For this agent, the groups represented 4 different times of exposure to an acidified 0.025% solution and a control group containing no protoveratrine.

RESULTS

The effects of different concentrations of colchicine and different times of exposure on nuclear mitotic division are listed in Table I and the statistical analysis of these data is shown in Table II. The analysis indicated that colchicine treatment significantly decreased the number of mitotically dividing nuclei ($F=5.18$; $P<0.01$) Further, the individual factors contributing to the total treatment effects (time, concentration, and interaction) all showed significant changes (ratios for the concentration and interaction factors were highly significant).

In Table I, examination of individual means (MNDN) and group means (GM) indicates that for the concentration factor alone (upper portion), all colchicine concentrations employed in this study decreased the total number of dividing nuclei as represented by the GM values when compared with the GM obtained for the control. Except for the 0.2% concentration, the GM values were significantly different than the control value. Except for the twelve-hour exposure to the 0.2% colchicine concentration, the MNDN values for exposure times of nine and twelve hours decreased as the concentration of colchicine increased ($P<0.05$ for the nine- and twelve-hour exposures to 0.4% and 0.8% colchicine). Also, for the colchicine concentrations of 0.4% and 0.8%, MNDN values decreased as the time of exposure was increased from three to twelve hours. There was little effect produced on MNDN values either by increasing the colchicine concentration for the three- and six-hour exposure times or by increasing the time of exposure at colchicine concentrations of 0.1% and 0.2%.

With time as the major variable (Table I, lower portion), MNDN values indicate more clearly that for exposure times of nine and twelve hours, a decrease in the number of dividing nuclei resulted as the concentration of colchicine was increased. A comparison of the differences between group means for the six-hour time exposure and any of the other times by the "Student" t test indicated that the differences were significant. The high GM value for the six-hour exposure time (3.55%) was primarily due to the high individual MNDN value of 5.3% obtained for the 0.8% colchicine concentration (also see Fig. 2, six-hour exposure time). Since this value failed to follow the general trend established for the other concentrations and exposure times, an additional experiment on roots exposed to 0.8% colchicine for a six-hour time period was indicated. Results obtained from an additional 20 allium bulbs showed the MNDN to be 3.2%; therefore, the 5.3% value obtained in the original experiment was assumed to be aberrant and for further graphical purposes (Fig. 2) the 3.2% value was used.

TABLE I.—EFFECTS OF COLCHICINE ON NUCLEAR DIVISION IN ALLIUM ROOTS

	Main Groups, Concentration															
	Control				0.1%				0.2%				0.4%			
Time, hr.→	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12
MNDN,[a] %	3.9	3.6	4.5	4.0	2.5	2.6	2.6	2.6	3.1	3.6	2.4	4.1	1.9	2.7	2.1	0.9
GM,[b] %	4.00				2.58				3.30				1.90			

	Main Groups, Time															
	Control				3 hr.				6 hr.				9 hr.			
Colchicine, %→	..	..	..	..	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8
MNDN,[a] %	3.9	3.6	4.5	4.0	2.5	3.1	1.9	2.4	2.6	3.6	2.7	5.3	2.6	2.4	2.1	1.5
GM,[b] %	4.00				2.48				3.55				2.15			

	Main Groups, Time															
	12 hr.															
Colchicine, %→	0.1	0.2	0.4	0.8												
MNDN,[a] %	2.6	4.1	0.9	0.9												
GM,[b] %	2.13															

[a] Mean number of dividing nuclei. [b] Group means.

TABLE II.—ANALYSIS OF VARIANCE[a] OF COLCHICINE EFFECTS ON NUCLEAR MITOTIC DIVISION IN ALLIUM ROOTS

Nature of Variation	Adjustment for Mean: Degrees of Freedom		9308.00: Sum of Squares		Mean Square		Experimental F Ratio	
Between treatments	19	..	508.31	..	26.753	..	5.18[b]	..
Different levels of concentration	..	4	..	235.29	..	58.823	..	11.37[b]
Different levels of time	..	3	..	44.80	..	14.933	..	2.89[c]
Interaction	..	12	..	228.22	..	19.018	..	3.68[b]
Error	80	..	413.73	..	5.172	..		
Total	99		922.04					

[a] Transformation of data to angles. [b] $P < 0.01$. [c] $P < 0.05$.

TABLE III.—EFFECTS OF VERILOID ON NUCLEAR DIVISION IN ALLIUM ROOTS

	Main Groups, Concentration															
	Control				0.1%				0.2%				0.4%			
Time, hr.→	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12
MNDN,[a] %	5.1	4.9	5.5	5.9	4.5	3.4	7.6	8.9	5.7	5.5	7.8	7.3	7.1	5.9	7.3	6.9
GM,[b] %	5.35				6.10				6.58				6.80			

	Main Groups, Time															
	Control				3 hr.				6 hr.				9 hr.			
Veriloid,[c] %→	..	..	..	..	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8
MNDN,[a] %	5.1	4.9	5.5	5.9	4.5	5.7	7.1	6.6	3.4	5.5	5.9	5.5	7.6	7.8	7.3	7.6
GM,[b] %	5.35				5.98				5.08				7.58			

	Main Groups, Time															
	12 hr.															
Veriloid,[c] %→	0.1	0.2	0.4	0.8												
MNDN,[a] %	8.9	7.3	6.9	6.7												
GM,[b] %	7.45															

[a] Mean number of dividing nuclei. [b] Group means. [c] Alkavervir (Veriloid) reference standard.

TABLE IV.—ANALYSIS OF VARIANCE[a] OF VERILOID EFFECTS ON NUCLEAR DIVISION IN ALLIUM ROOTS

Nature of Variation	Adjustment for Mean: Degrees of Freedom		20899.907: Sum of Squares		Mean Square		Experimental F Ratio	
Between treatments	19	..	239.309	...	12.595	...	2.46[b]	...
Different levels of concentration	..	4	...	42.854	...	10.714	...	2.09
Different levels of time	..	3	...	113.166	...	37.722	...	7.36[c]
Interaction	.	12	...	83.289	...	6.941	...	1.35
Error	80		410.198	...	5.128	...		
Total	99		649.507					

[a] Transformation of data to angles. [b] $P < 0.01$. [c] $P < 0.05$.

The effects of different concentrations of Veriloid and different times of exposure on nuclear mitotic division are listed in Table III and the statistical analysis of these data is shown in Table IV. The analysis indicated that, in contrast to the colchicine effects, Veriloid treatment significantly increased the number of mitotically dividing nuclei ($F=2.46$; $P<0.01$). Further, of the individual factors contributing to the total treatment effects, only the time factor indicated a significant change ($F=7.36$; $P<0.01$). Therefore, although the effects of both concentration and time were important for colchicine produced changes, the length of time in which roots were exposed to Veriloid appeared to be of

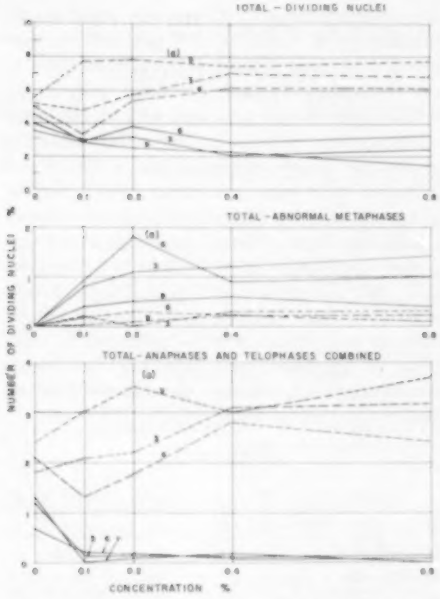
TOTAL – DIVIDING NUCLEI
TOTAL – ABNORMAL METAPHASES
TOTAL – ANAPHASES AND TELOPHASES COMBINED
NUMBER OF DIVIDING NUCLEI %
CONCENTRATION %

Fig. 2.—Effects of colchicine and Veriloid on nuclear division in *Allium* roots. (*a*), Hours of exposure; ——, colchicine; – – – Veriloid.

greater importance to nuclear mitotic division than were the concentrations employed.

Examination of the means in Table III indicates that for the concentration factor alone (upper portion) Veriloid, as employed in this study, had no significant effect on the total number of dividing nuclei when GM values were compared with the value obtained for the control.

With time as the major variable (Table III, lower portion), MNDN values indicate a general increase as the Veriloid concentrations are raised ($P<0.01$). The difference between GM values for the three-hour and twelve-hour exposure times, 5.98% and 7.45%, respectively, was significant ($P<0.05$). Further, the most marked increase in the total number of dividing nuclei occurred between the six- and nine-hour exposure times and the difference between these GM's was highly significant ($P<0.01$).

Figure 2 shows graphically the effects of colchicine and Veriloid on nuclear mitotic division in allium roots for three of the four exposure times studied; three, six, and nine hours. Effects produced by the twelve-hour exposure period were similar to the other exposure times and for purposes of simplification, they are not depicted. In general, the total number of dividing nuclei (upper graph) decreases with increasing concentrations of colchicine whereas for Veriloid, the dividing nuclei remained relatively unaffected (a slight but insignificant increase was the overall effect). The decrease in the total number of dividing nuclei at the concentrations of colchicine studied indicated that the agent was inhibitory at and above concentrations of 0.1%. Typical c-mitotic effects were produced by the colchicine. At the three-hour exposure time, the number of abnormal metaphases (middle graph) was observed to increase with each increase in colchicine concentration. Maximum effects were observed to occur at the three-hour exposure to 0.8% colchicine and at the six-hour exposure to 0.2% colchicine. At the three-hour exposure time to 0.8% colchicine, 1.4% of the total 2.5% of dividing nuclei showed abnormal metaphases (56% of the total number of dividing nuclei observed). For Veriloid, the "piling up" effect at metaphase, as noted with colchicine, was not observed.

In the course of investigating the effects of protoveratrine A on nuclear mitotic division, it became apparent that the changes produced were very nearly the same as those observed for Veriloid. For this reason, the investigation of protoveratrine A was not as extensive as it was for colchicine and Veriloid. The effects of different exposure times to 0.025% protoveratrine A on nuclear mitotic division are shown graphically in Fig. 3. The total number of dividing nuclei remained relatively unaltered by different times of exposure to the protoveratrine A. A slight but insignificant ($P>0.05$) increase over that observed for the control value may be the overall effect produced. The number of abnormal metaphases increased considerably for different times of exposure. At the six-hour exposure time, 1% of the observed nuclei showed abnormal metaphases in the same manner seen in the Veriloid study. Therefore, treatment of the allium roots with protoveratrine A produced mitotic nuclear changes although the number of mitotic dividing nuclei remained relatively unaffected.

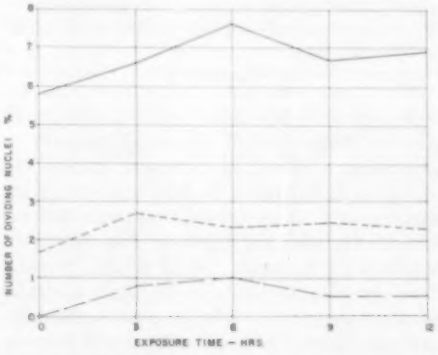

Fig. 3.—Effects of protoveratrine A on nuclear division in *Allium* roots. ——Total, dividing nuclei; – – – total, anaphases and telophases combined; — — — total, abnormal metaphases.

DISCUSSION

Colchicine, Veriloid, and protoveratrine A all produced polyploidy in allium roots, although, in general, the effects were much more pronounced after colchicine treatment. A comparison of the effects produced by the veratrum derivatives on nuclear mitotic division with those observed for colchicine revealed many interesting similarities and differences. The equatorial plate was completely absent in the colchicine-treated root tips, whereas

approximately 60% of all metaphases observed in the veratrum-treated root tips showed chromosomes in regular alignment in the equatorial region. It was impossible to differentiate between the two veratrum agents employed solely on the basis of the observable chromosome characteristics; however, the chromosomes of the veratrum-treated cells differed markedly from those treated with colchicine. Veratrum induced "sticky" or angled chromosomes and anaphase bridges were very prominent.

Colchicine treatment decreased significantly ($P < 0.05$) the overall number of dividing nuclei in cells, irrespective of concentration or time of exposure. Because this agent induces metaphasic arrest and yet has no effect on stopping initial mitotic activity, it probably indicates that the concentrations employed were too high to produce an optimum mitotic effect. The most marked differences between colchicine and the veratrum agents were noted with the anaphase and telophase stages (lower graph, Fig. 2). Colchicine treatment caused a great reduction, and sometimes a complete absence, of the number of anaphases and telophases. On the other hand, many anaphases and telophases were to be observed after treatment with the veratrums.

Thus, it appears that alkaloidal agents (mixtures or crystalline) from *Veratrum viride* Ait. and/ or *Veratrum album* L. having structural similarities to alkaloids obtained from sabadilla seeds (*Schoenocaulon officinale* A. Gray), may produce polyploidy in roots of *Allium cepa* by an action similar to that of veratrine (10). This action is markedly different and apparently inferior to that seen in colchicine-treated roots and suggests that the derivatives of veratrum do not exert their effects specifically on the mitotic apparatus *per se* as does colchicine, but possibly act by some modification of cytoplasmic function.

SUMMARY

An attempt has been made to determine whether some purified alkaloidal mixtures or crystalline alkaloids of veratrum possess the ability to modify the cycle of nuclear and cellular division, and to determine how such activity compares in kind and degree to that already established for colchicine. Root growth, preparation, and cell observations were accomplished by standard techniques. The per cent of cells in the process of nuclear division at the time of observation was calculated and results were subjected to statistical analysis. Drugs were tested in concentrations of 0.025 to 0.8 per cent for predetermined time periods varying from three to twelve hours. Typical colchicine mitotic (c-mitotic) effects were produced with all colchicine concentrations employed. Successful inhibition of the the spindle apparatus was achieved and polyploid cells were prominent. Both Veriloid and protoveratrine A produced frequent chromosomal aberrations although their appearance suggested a markedly different mode of action than that observed with colchicine. Polyploid cells were noted and the effects on nuclear mitotic division were similar to those produced by veratrine, an alkaloidal mixture whose constituents are structurally similar to those of the Veriloid and protoveratrine A employed in this study.

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The Effect of Anticholinergic Compounds on Several Components of Gastric Secretion in Pylorus-Ligated Rats*

By MORTON E. GOLDBERG† and G. VICTOR ROSSI

A study was made of the effect of seven anticholinergic compounds: atropine, diphenmethanil (Prantal), oxyphenonium (Antrenyl), propantheline (Pro-Banthine), tridihexethide (Pathilon), α-(p-dimethylaminocyclohexyl)benzhydrol salicylate (SCH 3085), and 1-methyl-4-piperidyl benzilate methobromide (SCH 3444), on various factors which have been cited as bearing a relationship to peptic ulcer (gastric fluid volume, acidity, proteolytic activity, lysozyme activity, and mucin content of gastric secretion, and uropepsin activity). The levels of these several components were contrasted in four-hour ("normal") and fifteen-hour (ulcerated) pylorus-ligated rats. Equipotent antisecretory doses of the anticholinergic drugs significantly reduced the proteolytic activity and free acidity, and increased the lysozyme activity and mucin content per ml. of gastric fluid, but did not alter uropepsin activity in four-hour ligated rats. Comparable, but less consistent results were obtained in ulcerated (fifteen-hour) animals.

NO RECOGNIZED single concept satisfactorily explains the development of peptic ulcer. In addition to nervous and psychic influences, which are believed to play a major role, muscle spasm, ischemia, mechanical irritation, decreased tissue resistance, and abnormal levels of the constituents of gastric secretion have been proposed as factors which contribute to erosion of the mucosa and propagation of the ulcer.

Hydrochloric acid and pepsin have frequently been implicated as agents involved in the formation and maintenance of chronic peptic ulcer. Thus, drugs which possess antisecretory or antipepsin effects, or both, are regarded as major adjuncts in the treatment of this condition. In addition, abnormal levels of lysozyme (1, 2) and uropepsin (3, 4) activity have been considered of diagnostic or pathognomonic importance in peptic ulcer.

In this study an attempt was made to correlate certain biochemical components, present in gastric juice or urine, which have been considered as bearing a relationship to the etiology of peptic ulcer. The investigation basically included a determination of the effects of several anticholinergic compounds upon the following factors of the gastric juice of pylorus-ligated rats: volume, acidity, proteolytic activity, lysozyme activity, and mucin content. In addition, the excretory rate of the urinary proteolytic enzyme, commonly referred to as uropepsin, was determined.

Five commercially available anticholinergics: atropine, diphenmethanil, oxyphenonium, propantheline tridihexethide, and two experimental compounds: α-(p-dimethylaminocyclohexyl)-benzhydrol salicylate (SCH 3085),[1] and 1-methyl-4-piperidyl benzilate methobromide (SCH 3444),[1] were utilized in this evaluation.

EXPERIMENTAL

Animals and Experimental Design.—Male albino Sherman rats (Scott Farms), maintained on Rockland complete rat pellets and water, *ad libitum*, and housed in temperature and humidity controlled quarters, were used throughout these studies. Prior to ligation of the pylorus, all animals were fasted for forty-eight hours but permitted free access to water. During the fasting and experimental periods, the animals were kept in individual metabolism cages designed to minimize coprophagy and avoid contamination of urine with fecal matter. Only those animals weighing between 185 and 215 Gm. after fasting were utilized.

The technique employed for collection of gastric fluid was essentially that described by Shay (5). The data for "normal" animals presented in this report are based on values obtained with the four-hour pylorus-ligated rat. According to Shay (5), evidence of ulceration was observed only once in a series of 300 four-hour pylorus-ligated rats. In this study of more than 700 similar preparations, gastric ulceration was never observed; therefore, for the purpose of this report, the term "normal" appears justified.

Extension of the ligation period results in ulceration of the stomach and the degree of ulceration is related to the duration of the ligation period. Prolonged ligation ultimately results in perforation of the stomach and death of the animal. The data for "ulcerated" animals given in this report are based on values obtained with the fifteen-hour pylorus-ligated rat. This interval was selected on

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† Present address: Mellon Institute, Pittsburgh, Pa.

[1] Generously supplied by Schering Corp., Bloomfield, N. J.

the basis of preliminary experiments which indicated that periods of less than fifteen hours were not uniformly associated with ulcer formation, whereas ligation for longer intervals resulted in excessive incidence of perforated ulcers, with consequent loss of gastric fluid.

Following sacrifice of the animals the esophagus was grossly examined for evidence of ulceration, the stomach was incised and its contents collected in a graduated centrifuge tube. The mucosal surface of the stomach removed from each fifteen-hour ligated rat was examined with the aid of a stereoscopic (wide-field binocular) microscope equipped with an eyepiece micrometer. The "degree of ulceration" was estimated by a modification of the characterization suggested by Keyrilainen and Passonen (6), an evaluation which takes into consideration both the number and size of the ulcers. This system is outlined in Table I.

TABLE I.—ULCER SCORING SYSTEM

Numerical Rating	Ulcer Score[a]	Descriptive Rating
0	0	Normal
1	Hemorrhagic	Irritation
2	Less than 4	Minimal ulceration
4	4 to 9	Moderate ulceration
6	10 to 19	Moderately severe ulceration
8	20 to 39	Severe ulceration
10	More than 39 or perforated	Very severe ulceration

[a] Ulcer score = total number of ulcers (gastric and esophageal) + total length of all ulcers in mm. (greatest dimension of each ulcer).

A study was undertaken to determine the relative activity of the seven anticholinergic compounds utilized in this investigation on the basis of reduction of the volume of gastric secretion. The intention was to establish "equipotent" antisecretory doses of these compounds in order that one of the factors being considered (i. e., volume of gastric secretion) would remain relatively stable throughout the investigation. The average volume of gastric fluid collected from four-hour pylorus-ligated rats following administration of several different doses of each of the seven anticholinergic drugs was compared to the average volume secreted by control animals which received an injection of a comparable volume of saline solution. All of the compounds utilized were dissolved in equal volumes of physiological saline and injected subcutaneously immediately after ligation of the pylorus. The slope of each dose-response curve indicated a linearity in response and no lack of parallelism among the various agents studied. The calculated equipotent doses (Table II), which were used in all subsequent studies, resulted in a volume of gastric secretion equivalent to 37–39% of the control volume. It should be noted that these doses may be considered "equipotent" only under the conditions of this investigation (i. e., four-hour pylorus-ligated rats of specified sex, weight, strain, etc.).

Analytical Procedures.—Acidity of the gastric samples was determined by titration with 0.02 *N* sodium hydroxide, using dimethylaminoazobenzene (Topfer's reagent) and phenolphthalein as indicators for free and total titratable acidity, respectively. Measurements of pH were made with a Beckman pH meter.

TABLE II.—APPROXIMATE EQUIPOTENT DOSES OF ANTICHOLINERGIC DRUGS BASED ON VOLUME OF GASTRIC SECRETION IN FOUR-HOUR PYLORUS-LIGATED RATS

Compound	Dose, s. c., mg./Kg.	Number of Animals	Volume of Gastric Secretion, ml. ± S. D.
Control (saline)	...	122	5.85 ± 0.74
Atropine	10.0	30	2.22 ± 0.35
Oxyphenonium	1.0	30	2.25 ± 0.35
Tridihexethide	1.0	30	2.27 ± 0.48
Diphenmethanil	15.0	40	2.28 ± 0.36
Propantheline	0.05	40	2.31 ± 0.63
SCH 3085	2.0	30	2.23 ± 0.41
SCH 3444	1.0	40	2.29 ± 0.37

The method of proteolytic analysis employed in this study has been described previously (7). It represents an adaptation of Wesselman's (8) modification of the Anson and Mirsky (9) procedure, which essentially consists of quantitative proteolytic digestion of hemoglobin by pepsin. Results are expressed in milligrams equivalent to N. F. X Reference Pepsin.

Lysozyme activity was measured by a method previously described (10) which represents a modification of the bacteriolytic turbidometric procedures of Lobstein and Fogelson (11) and Smolelis and Hartsell (12). Employing crystalline lysozyme[2] from egg white as a provisional standard, results are obtained in micrograms of lysozyme.

Uropepsin determinations were performed by a method based upon the procedure of West, *et al.* (13). The technique basically consists of the precipitation of casein particles by a sample of activated urine containing the enzyme. Results were calculated according to the procedure of Hollander (14) and are expressed in units of uropepsin excreted per hour; one unit of activity being equivalent to 0.26 mcg. of crystallized pepsin (Armour) (15).

A colorimetric procedure for the determination of gastric mucin was adapted from the method of Glass, *et al.* (16, 17). Gastric samples were fractionated into three components, each with chromogenic activity. Gastric mucin is observed in both solid ("mucoid") and liquid ("mucoprotein" and "mucoproteose") fractions of gastric juice. Results are expressed as mg. of mucin in each fraction or in the entire sample.

RESULTS AND DISCUSSION

Four-Hour Pylorus-Ligated Rat Study.—Determination of "equipotent" antisecretory doses in the four-hour pylorus-ligated rat indicated that, on a weight basis, considerable differences in antisecretory potency existed among the seven anticholinergic compounds evaluated (Table II). Measurement of several components of gastric secretion collected from pylorus-ligated rats four hours after administration of approximately equi-

[2] Kindly supplied by Armour Laboratories, Chicago, Ill.

potent antisecretory doses revealed a considerable specificity of action among the limited series of anticholinergic drugs employed in regard to the factors (gastric fluid volume, acidity, proteolytic activity, lysozyme activity, mucin content, and uropepsin activity) investigated.

In doses which uniformly reduced the volume of gastric fluid secreted during a four-hour period in pylorus-ligated rats to 37 to 39% of the control volume (i. e., approximate equipotent doses), all of the anticholinergic agents studied, with the exception of propantheline, significantly reduced proteolytic activity. Figure 1 indicates the mean proteolytic activity of gastric samples collected from anticholinergic treated rats in terms of per cent of the control value (i. e., data obtained from saline treated rats) on a concentration (mg./ml.) basis. It is evident that the per cent reduction in the total amount of enzyme present in the gastric secretion was considerably greater than that presented in Fig. 1, inasmuch as the volume of gastric fluid secreted during the experimental period was markedly inhibited.

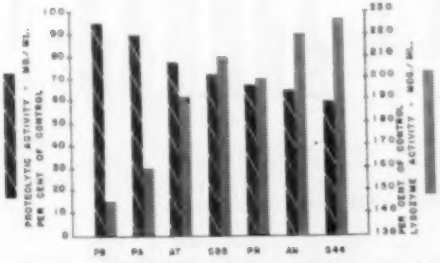

Fig. 1.—Proteolytic activity and lysozyme activity in the four-hour pylorus-ligated rat. PB, propantheline; PA, tridihexethide; AT, atropine; S85, SCH 3085; PR, diphenmethanil; AN, oxyphenonium; S44, SCH 3444.

The total lysozyme activity of the gastric juice collected from control rats ($n = 30$) after a four-hour period of ligation was found to be equivalent to 3.05 ± 1.18 mcg. of the enzyme. Administration of the anticholinergic compounds produced, in all cases, a significant reduction in the total content of lysozyme, but an elevation in the concentration of lysozyme in gastric fluid. These increases were in the range of 145–225% of normal levels of activity per ml. of gastric juice (Fig. 1).

Gastric fluid obtained from four-hour pylorus-ligated rats which were not treated with anticholinergic drugs exhibited a pH of 1.35 ± 0.25. All seven anticholinergic compounds raised this value somewhat (average of pH 2.0), however, there were no obvious quantitative differences in this respect among the individual drugs. The anticholinergic drugs uniformly reduced the concentration of free gastric acidity (Fig. 2) to approximately one-half of the control value, which corresponded to a decrease in free acidity in the entire gastric fluid volume to 15–20% of the control level.

None of the anticholinergic compounds significantly altered the uropepsin activity of urine collected during the four-hour study (Fig. 2). Clinical reports are contradictory in regard to the effect of such compounds on uropepsin levels (18, 19). The amount of pepsinogen which enters the plasma appears to remain relatively constant, even after the administration of drugs which markedly reduce gastric pepsin activity. It would appear that none of the drugs, in the doses used, have a marked inhibitory effect on the endocrine activity of the chief cells; rather they appear to preferentially inhibit the exocrine function of these cells.

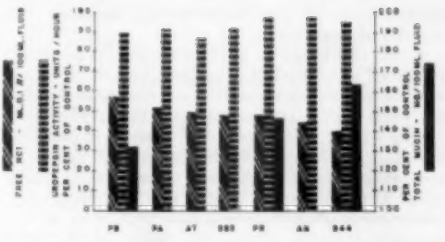

Fig. 2.—Free acidity, uropepsin activity, and total gastric mucin concentration in the four-hour pylorus-ligated rat. PB, propantheline; PA, tridihexethide; AT, atropine; S85, SCH 3085; PR, diphenmethanil; AN, oxyphenonium; S44, SCH 3444.

Of the three drugs studied for their effect on gastric mucin levels; propantheline, diphenmethanil, and SCH 3444; the latter compound produced the greatest increase (approximately 60% greater than the control level) in the concentration of gastric mucin (Fig. 2). However, a marked decrease was observed in the total amount of mucin secreted during the four-hour experimental period. This biphasic effect on concentration and total secretion of mucin, probably related to the inhibition of gastric fluid volume, was similar to that observed in regard to lysozyme activity. It has been suggested that both gastric mucoid and lysozyme are derived from the surface epithelial cells of the gastric mucosa, therefore it is conceivable that changes in the level of one of these components would be reflected by similar changes in the concentration of the other.

Within the series of anticholinergic drugs studied, a positive correlation was found to exist between the reduction of free acidity and the decrease in proteolytic activity of the gastric juice obtained from four-hour pylorus-ligated rats. An inverse relationship was observed between the effects of the anticholinergic agents on the proteolytic and lysozyme concentrations of gastric secretion. Lysozyme titer was found to be elevated to the greatest extent by those drugs which produced the greatest decreases in proteolytic activity. These effects appear to be partly attributable to the level of gastric acidity; a decrease in acidity being associated with a decrease in peptic activity (optimum pH 1.5–2.2) and a concomitant increase in lysozyme activity (optimum pH approximately 6).

Fifteen-Hour Pylorus-Ligated Rat Study.—All animals in this study were grouped into one of seven "ulcer classes" on the basis of ulcer score (Table I). Results of the analyses of the several components of gastric secretion (fluid volume, acidity, proteolytic activity, lysozyme activity, mucin content) were

evaluated on the basis of both drug treatment and severity of ulceration.

Doses of the seven anticholinergic drugs which produced approximately the same degree of inhibition of gastric secretion in the four-hour ligated rat were not "equipotent" in the fifteen-hour ligated animal, due largely to differences in duration of activity. Marked differences in the extent of protection provided against ulceration of these drugs were also noted (Table III). Although the lowest mean ulcer score was associated with the experimental group having the least gastric fluid volume and the highest ulcer scores were associated with groups evidencing the greatest volumes of gastric secretion, there was an inconsistent relationship among the remaining drug treated groups in regard to gastric fluid volume and severity of ulceration. However, if results are considered on the basis of ulcer response, without regard to drug treatment, there appears to be positive correlation between gastric fluid volume and the degree of ulceration produced by the experimental procedure employed in this study (Fig. 3).

TABLE III.—INHIBITION OF GASTRIC SECRETION AND ULCERATION BY ANTICHOLINERGIC DRUGS IN THE FIFTEEN-HOUR PYLORUS-LIGATED RAT

Compound	Dose, s. c., mg./Kg.	n/N[a]	Volume of Gastric Secretion, ml. ± S. D.	Ulcer Score[b]
Control (saline)	...	25/30	13.57 ± 1.67	6.9
Atropine	10.0	10/10	10.53 ± 1.87	3.4
Oxyphenonium	1.0	9/10	9.24 ± 2.78	2.4
Tridihexethide	1.0	10/10	11.16 ± 1.89	2.9
Diphenmethanil	15.0	10/10	4.20 ± 1.03	0.0
Propantheline	0.05	10/10	10.21 ± 2.32	1.6
SCH 3085	2.0	9/10	13.23 ± 1.96	4.5
SCH 3444	1.0	10/10	8.58 ± 1.49	1.9

[a] Number of nonperforated animals/total number of animals employed. [b] Refer to Table I.

The effect of the anticholinergic agents on proteolytic activity was less evident in the fifteen-hour study than in the four-hour experiments. Only three compounds (tridihexethide, SCH 3085, and SCH 3444) significantly reduced proteolytic activity per unit volume of gastric secretion. Conceivably, the inhibitory effect of the anticholinergic drugs on peptic activity may terminate before their inhibitory influence on gastric fluid secretion. On the basis of grouped ulcer score, a definite correlation was demonstrated between the total proteolytic activity of the gastric fluid and severity of ulceration (Fig. 3). Again, the significantly greater fluid volume exhibited by the high ulcer score groups contributed markedly to this quantitative difference.

Considerable variation in the lysozyme concentration of gastric fluid was observed among the anticholinergic treated groups in the fifteen-hour study. In general, lower levels of lysozyme activity were found in ulcerated than in nonulcerated animals but no consistent relationship could be demonstrated between lysozyme concentration and severity of ulceration (Fig. 3).

The highest free acid concentrations were found in the secretions of those stomachs exhibiting

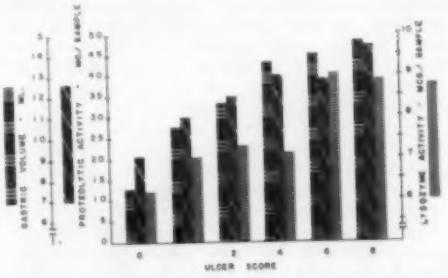

Fig. 3.—Comparison of gastric fluid volume, proteolytic activity, and lysozyme activity in the fifteen-hour pylorus-ligated rat on the basis of "ulcer score" (see Table I).

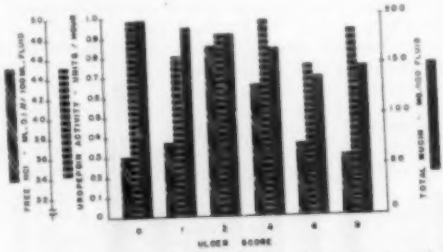

Fig. 4.—Comparison of free acidity, uropepsin activity, and total gastric mucin concentration in the fifteen-hour pylorus-ligated rat on the basis of "ulcer score" (see Table I).

moderate ulceration, whereas lower acid concentrations were associated with both lower and higher ulcer scores (Fig. 4). It is probable that neutralization of gastric acid by blood and tissue debris at the sites of ulceration may have been partly responsible for the reduced acid concentration observed in the severely ulcerated animals.

As was noted in the results of the four-hour experiments, the seven anticholinergic drugs in the doses used, did not significantly alter the uropepsin activity of urine secreted during a fifteen-hour period of pylorus ligation. Furthermore, no relationship was observed between the degree of gastric ulceration produced by this technique and the levels of uropepsin activity (Fig. 4).

Prolongation of the period of pylorus-ligation from four to fifteen hours resulted in an average increase of 70% in the gastric mucin concentration of control (saline-treated) animals as contrasted to an average increase of only 25% in the anticholinergic treated groups. Since a large percentage of the increase in mucin concentration was due to elevated mucoprotein levels, it is not improbable that the increased amounts of gastric fluid associated with the extended period of ligation may have "washed out" preformed, but as yet nonsecreted, mucoprotein from the mucous neck cells. The highest mucin concentration was associated with the group evidencing minimal mucosal damage ("0" score). As the degree of ulceration increased, a decrease in mucin concentration was observed (Fig. 4). However, increases in the severity of

ulceration were accompanied by progressive increases in total mucin content of the gastric samples, apparently related to the higher average fluid volumes characteristically found in the more severely ulcerated animals.

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Antithixotropic Behavior of Magnesia Magma*

By CLIFFORD W. CHONG, STUART P. ERIKSEN, and JOSEPH V. SWINTOSKY

An unusual rheological behavior which involves a sol-gel transformation during measurement in a rotational viscometer, and a spontaneous return to a sol state on standing, has been observed in magnesia magma U. S. P. The phenomenon is the reverse of thixotropy. Thus, the term "antithixotropy" perhaps best describes this rheological behavior. This unique, seldom-encountered flow property was detected at shear rates of approximately 30 reciprocal seconds and greater, using a Drage rotating bob rheometer, and later confirmed with a modified Stormer viscometer. At rates of shear less than 30 reciprocal seconds, the material exhibited pseudoplastic flow. Rheograms of the data suggest that the increase in consistency during shear is due primarily to an increase in the apparent yield value with no significant change in the slopes of the flow curves. The result is a system which has good suspending characteristics, and one which, at the same time, is easily pourable. Equations analogous to those used for thixotropic and pseudoplastic flows are presented to describe the rate at which the resistance to flow increases with duration of shear, and the rate of decrease in the apparent viscosity with increasing rates of shear. A description of the Drage Viscostructure rheometer is also presented.

THIS PAPER reports the unique rheological behavior of magnesia magma U. S. P. Magnesia magma thickens when subjected to shear; on standing, it returns to a fluid state. Flow behavior of this type has been reported by other investigators (1–5) and has been designated "antithixotropy" (4) or "negative thixotropy" (2). Antithixotropy, heretofore found only in fluid polymer and pigment systems, has not been previously reported in pharmaceutical systems.

The purpose of this study was to characterize the antithixotropic behavior of magnesia magma in terms of parameters similar to those used to describe thixotropic and pseudoplastic flows.

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EXPERIMENTAL

Apparatus.—The Drage Viscostructure rheometer and a modified Stormer viscometer were used in this study. The Drage rheometer, a fairly recent development, operates on the rotating bob-precision spring principle. It is capable of yielding data in fundamental units. The instrument consists essentially of a freely suspended synchronous motor, a calibrated precision spring, and four sets of measuring cups and bobs. The motor drives the measuring bob which hangs concentrically in the stationary measuring cup. The test fluid is placed in the annulus between the cup and bob, and exerts a frictional force when the bob is rotated. This force causes the motor to be twisted backwards against the calibrated precision spring until the resistance encountered by the bob is the same as the counterforce which results from the tension of the spring. The degrees through which the motor is turned are indicated on a dial, and are proportional to the fric-

tional resistance exerted by the liquid on the bob. The instrument is capable of nine different speeds through the use of mechanical (gear ratio) and electrical (frequency modulation) changes. The rates of shear range from 4 to 1,600 reciprocal seconds, depending upon the cup and bob used.

The Stormer viscometer was modified only to the extent of making a new cup and bob. The cup had a diameter of 3.18 cm. and was 5.45 cm. in height, while the bob was 2.54 cm. in diameter and 3.90 cm. in height.

Materials.—Magnesia magma U. S. P. was studied. Samples prepared in our laboratory were made by (*a*) the U. S. P. X method[1] and (*b*) diluting a 30% Hydro-Magma[2] of magnesium hydroxide to 7.5% to conform to U. S. P. specifications. Commerical samples of magnesia magma, aluminum hydroxide gel U. S. P., and bismuth magma N. F. were also tested, the latter two for comparison only.

Preparation and Prehandling of Test Samples.—The test samples were prepared by placing the thoroughly mixed magma in round 120-ml. wide-mouth bottles. The bottles were capped and the samples were stored at room temperature for seven days. It was found that rheograms made daily showed no further change after the sixth day of storage, indicating that the magma had attained structural equilibrium in this time.

Immediately before measurement on the viscometer, each bottle of magma was placed tangentially on a horizontally rotating shaft (1-in. diameter), and turned end over end at 44 r. p. m. for exactly one minute. The magma was then transferred to the Drage measuring cup. The measuring bob was gently inserted, and the assembly placed in position on the Drage instrument, in a thermostatically controlled water bath set at 25° ±0.2°. The viscometer was turned on after exactly fifteen minutes had elapsed from the time the magma was first agitated on the rotating mixer.

Rheological Measurements With the Drage Rheometer

The Antithixotropic Rheogram.—The general scheme for obtaining the data for the antithixotropic rheogram was to apply increasing rates of shear up to maximum value, followed by decreasing rates of shear. The time of shear at each shear rate was held constant while making both the up- and downcurves, i.e., the total time allowed for decreasing the rate of shear from the maximum shear rate was the same as the time used to increase it to this point.

The Effect of Time at Constant Shear.—Six separate samples were run in related manners, the only difference being the duration of shear at a constant initial rate of shear. The procedure is best visualized by reference to Fig. 2. The measurement of each sample was started at shear rate D_a which, in this case, was selected as the constant initial rate of shear. Sample 1 was sheared at rate D_a for fifteen seconds, then at rate D_b for fifteen seconds, and finally at rate D_c for fifteen seconds, yielding the shearing stresses a_1, b_1, and c_1, respectively. Sample 2 was sheared an additional fifteen seconds at rate D_a or a total of thirty seconds, then at rates D_b and D_c in fifteen-second intervals. For each succeeding sample, the duration of shear at rate D_a was increased fifteen seconds beyond that of the previous sample before obtaining the shearing stress points of the upcurve at rates D_b and D_c.

Shear rates D_a, D_b, and D_c shown in Fig. 2 corresponded to values of 344, 515, and 688 reciprocal seconds, respectively. Initial rates of shear ranging from 9.1 to 515 reciprocal seconds were employed in other measurements.

The Effect of Shear at Constant Time.—The upcurves of the rheograms for five magnesia magma samples were obtained as described above in "The Antithixotropic Rheogram." For each succeeding sample, the duration of shear at each shear rate was increased by fifteen seconds. Thus, the first curve was made using fifteen seconds at each shear rate, the second using thirty seconds, etc.

Rheological Measurements with the Stormer Viscometer

The Antithixotropic Rheogram.—In making the upcurve with the Stormer viscometer, the sample was first sheared with a net force of 26.4 Gm. and the time for 100 revolutions of the bob was recorded. A 2-Gm. weight was immediately added to the weight pan and the same sample was once more subjected to shear under this increased force. The procedure was repeated until, in addition to the 26.4 Gm., 14 Gm. had been added in 2-Gm. increments. Immediately after the final measurement of the upcurve, the procedure was reversed to form the downcurve, i.e., 2 Gm. was removed from the weight pan after each 100 revolutions of the bob. As a result, shear rate values were obtained at corresponding shearing stresses on both the up- and downcurves.

The Effect of Time at Constant Shear.—The sample was sheared with a net force of 25.4 Gm., and the time for 100 revolutions of the bob measured. The measurement was repeated several times using the same sample and the same shearing force until no further change in the time for 100 revolutions was noted.

RESULTS AND DISCUSSION

The unique characteristic of magnesia magma is its reaction to shear. A pronounced increase in consistency may be induced by subjecting the material to a constant rate of shear greater than thirty reciprocal seconds. The degree of thickening depends upon the duration of shear, and, also, upon the magnitude of the stress applied. When sheared alternately with increasing followed by decreasing rates of shear, the material continues to thicken, but at a decreasing rate, until an equilibrium is reached (Fig. 1). At this stage the system is pseudoplastic, and is characterized by a high apparent yield value. Note, however, that the slope at any point along the pseudoplastic flow curve is practically the same as the slopes of the preceding upcurves at the corresponding rates of shear. On standing, the material spontaneously returns to a sol state characteristic of its structure prior to shearing.

Since the flow curves for magnesia magma were dependent upon both duration and rate of shear, it was desirable to use a standard procedure for pre-

[1] This method is also described in Remington's Practice of Pharmacy, 11th ed., Mack Publishing Co., Easton, Pa., p. 488.

[2] Produced by Merck and Co., and distributed by Whittaker, Clark, and Daniels, Inc., New York 13, N. Y.

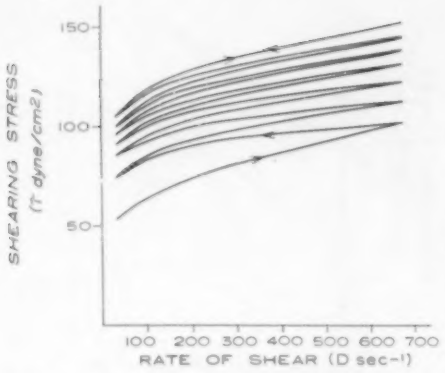

Fig. 1.—Rheograms showing the effect of repeated rheological measurements on magnesia magma.

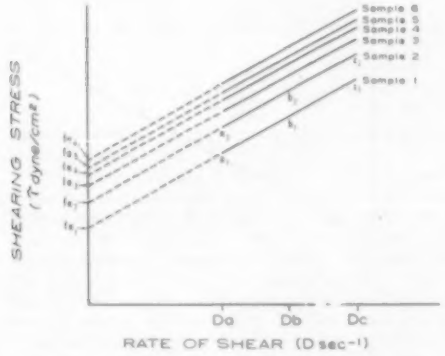

Fig. 2.—Rheograms showing the effect of duration of shear on magnesia magma at a constant rate of shear (Da).

paring and prehandling the test samples before the viscosity measurements were made. The data obtained by the proposed method were reproducible and comparable from one measurement to another.

The magnesium hydroxide suspensions of this study gave rheograms of the type shown in the first cycle of Fig. 1. Several points of interest can be noted from an examination of the flow curve.

The upcurve is similar to the flow curve of a typical pseudoplastic substance. As the rate of shear is raised, the increase in resistance to shear, τ (6), per unit increase in the shear rate, D, approaches a constant value. Consequently, the upper portion of the curve approaches a straight line asymptotically. After the top rate of shear has been reached, the succeeding shearing stress measurements along the downcurve assume values larger than those on the upcurve at the corresponding rates of shear.

If, after the downcurve is completed, the rate of shear is promptly increased again, the points along the upcurve will be greater than those previously obtained at the same shear rates. The points along the subsequent downcurve will be even larger than those found earlier. Successive up- and downcurves will yield larger and larger shearing stress values at each rate of shear. The rate of increase in shearing stress diminishes, however, until an equilibrium is established where both the up- and downcurves coincide as shown in Fig. 1. At this equilibrium the system is gel-like and possesses the unusual ability to suspend large, dense particles while still being readily pourable. For instance, glass beads, 1-mm. in diameter and having a density of 2.5 appear to remain suspended as long as the system is in this gel state. As the system spontaneously reforms its sol structure on standing, the glass beads slowly settle.

The Effect of Time at Constant Shear.—The effect that duration of shear has upon the resistance of magnesia magma to shear is shown in Fig. 2. The figure represents upcurves made on samples each of which had been subjected to increasing durations of shear at a constant initial shear rate indicated by D_a. The straight line portions of the upcurves, connecting the points a, b, and c, show the parallel relationship between the slopes of the curves; also, extrapolation of the lines to the shearing stress axis indicates the apparent yield values, f_a. The qualification "apparent" is intended to indicate that the measured yield values vary with rate of shear.

A linear relation results when the shearing stress, τ (or the apparent yield value, f_a) is plotted against the logarithm of the time, t, at shear rate D_a. This is shown in Fig. 3. The relation between the change in shearing stress (or apparent yield value) and the duration of shear, then, may be expressed similarly to data of thixotropic flow systems (7).

$$R = \frac{\tau_2 - \tau_1}{\log t_2/t_1} \quad \text{or} \quad = \frac{f_{a_2} - f_{a_1}}{\log t_2/t_1}$$

where R is the constant which describes the interrelationship between shearing stress (or apparent yield value) and duration of shear, and might be termed the coefficient of increased resistance to shear with time. The magnitude of the constant R is a function of the constant rate of shear used, D_a, as indicated by the change in the slopes of the τ *versus* log t plots at various values of D_a.

It is interesting to note that the increase in shearing stress occurs only at rates of shear greater than

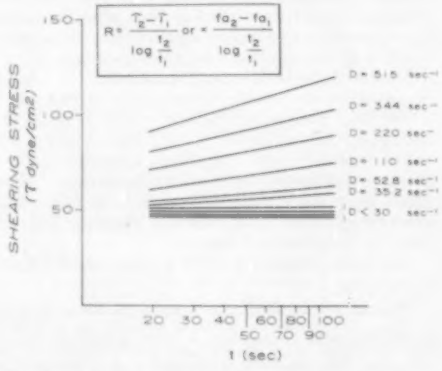

Fig. 3.—Increased resistance to shear of magnesia magma as a function of the rate of shear.

approximately thirty reciprocal seconds. Below this value, the effect of duration of shear is apparently nil. A rheogram of the data obtained at shear rates less than thirty reciprocal seconds results in a typical pseudoplastic flow curve as shown in Fig. 4.

In Fig. 5, values of log R obtained above shear rates of thirty reciprocal seconds are plotted *versus* log D. This plot yields a straight line which describes how the resistance to shear increases with time at various constant rates of shear.

The Effect of Shear at Constant Time.—Although there is an increase in the resistance to shear with time when magnesia magma is subjected to a constant rate of shear, there is, in addition, a decrease in the apparent viscosity with increasing rates of shear as illustrated in Fig. 6. Analogous to pseudoplastic systems (8), a straight line is obtained when the log of apparent viscosity,[1] η_a, is plotted against the log of shear rate, D (Fig. 7). The apparent viscosity in this case, is defined by the equation

$$\eta_a = \tau/D$$

which describes the slope of the line connecting the point (τ, D), on the upcurve of the rheogram, with the origin. The relation between the apparent viscosity and the rate of shear corresponding to it on the upcurve may be described by the equation

$$S = \frac{\log \eta a_1/\eta a_2}{\log D_2/D_1}$$

where S is the coefficient of decreasing apparent viscosity with increasing rates of shear. Figure 7 indicates that the constant S is relatively independen of the duration of shear at each shear rate.

It should be noted that the exact shape and position of the up- and downcurves, at constant durations of shear, will be governed in considerable measure by the instrument and the rates of shear chosen for the rheological measurements. The constants R and S, therefore, as determined in this study are not absolute valnes assignable to magnesia magma, but are rather relative values for determining the influence of (*a*) a constant rate of shear on shearing stress when the duration of shear is the variable, and (*b*) a variable rate of shear on shearing stress when the duration of shear at each shear rate is constant. By using this experimental approach, it has been possible to characterize the rheological behavior of magnesia magma, in a range of shear rates from nine to six hundred and eighty-eight reciprocal seconds, in terms of recognized rheological parameters.

Studies with the Stormer Viscometer.—It seemed desirable to use another viscometer to determine if the data obtainable from it would confirm the antithixotropic behavior of magnesia magma observed in studies with the Drage instrument. The Stormer viscometer was used for this purpose. Data which show the increased resistance to shear with duration of shear at a constant shearing force of 25.4 Gm. are given in Table I.

The rates of shear in Table I were calculated ac-

[1] Normally, for pseudoplastic systems the log of shearing stress, τ, rather than the apparent viscosity, η_a, is plotted against the log of shearing rate, D, yielding a straight line of slope > 1. This plot yields a straight line for antithixotropic data also. Since, however, we desired to show the decrease in the ability of magnesia magma to resist flow with increasing rates of shear, it was felt that the apparent viscosity plot was more illustrative.

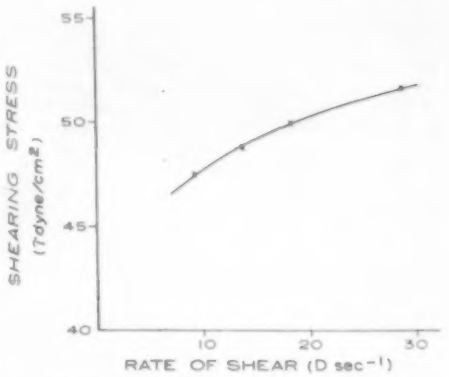

Fig. 4.—Rheogram of data obtained at rates of shear less than 30 sec.$^{-1}$.

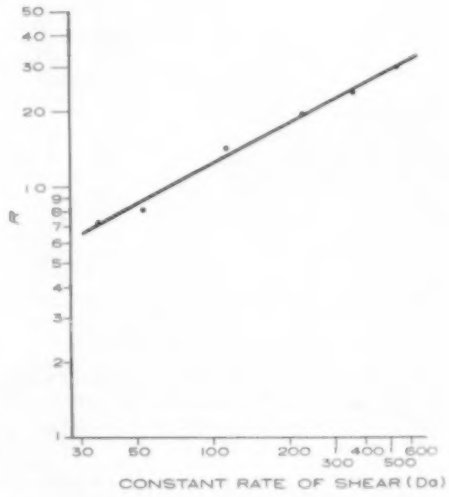

Fig. 5.—Plot of log constant rate of shear (Da) *vs.* log R.

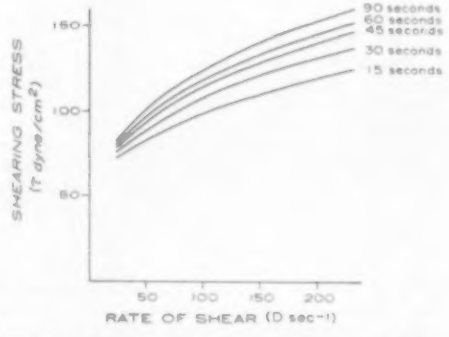

Fig. 6.—Rheograms showing the effect of duration of shear upon the upcurve.

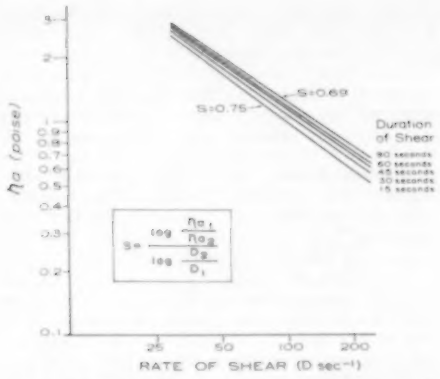

Fig. 7.—Plots of log η_a *vs.* log D showing the rates at which the apparent viscosity decreases with increasing rates of shear at various durations of shear.

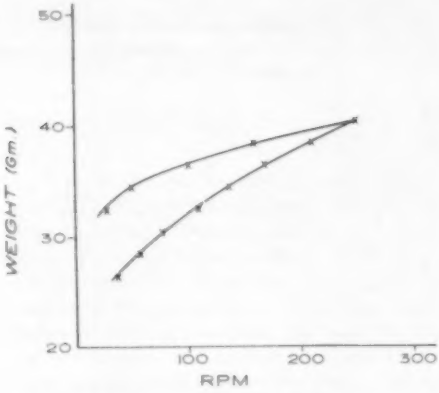

Fig. 8.—Stormer rheogram for magnesia magma.

cording to the method described by Reiner and Riwlin (9).

$$D = \frac{2\,(\text{r. p. m.})\,R_c}{9.55\,(R_c^2 - R_b^2)}$$

where R_c and R_b are the radii of the measuring cup and bob, respectively.

The gradual increase in the time of 100 revolutions of the bob indicates that magnesia magma increases in consistency when it is sheared repeatedly with a constant shearing force. After the seventh trial there is no further change in the time. This may be due to either or both of two things: (*a*) the system has attained structural equilibrium, or (*b*) since the rate of shear has decreased to less than thirty reciprocal seconds no further increase in consistency will occur.

Stormer viscometer data which show the antithixotropic behavior of magnesia magma are given in Table II. A rheogram of the data is shown in Fig. 8.

The Stormer viscometer operates only at selected shearing stresses. As a result, the duration of shear varies at each rate. Despite this inability to maintain a constant duration of shear, as one can with the Drage rheometer, the data obtained with the Stormer viscometer can be plotted to show the typical flow curves of time-shear dependent substances. Consequently, the Stormer rheogram in Fig. 8 shows the characteristic antithixotropic flow curve as the Drage rheogram in the first cycle of Fig. 1.

The phenomenon of antithixotropy is seldom encountered in pharmaceutical systems. Even among the suspensions chemically related to magnesia magma, such as aluminum hydroxide gel U. S. P. and bismuth magma N. F., we failed to find signs of such behavior. Instead, these substances were thixotropic. A comparison of the flow curves of these substances with that of magnesia magma is shown in Fig. 9.

Although antithixotropy bears some resemblance to dilatancy and also to rheopexy, it nevertheless differs from them in several important respects. The rheological behavior of antithixotropic systems is dependent upon the duration as well as the rate of shear. Dilatant systems are dependent upon rate of shear only. Rheopectic systems, i. e., those which gel more rapidly when a small shear is applied than when at complete rest (10), are gels at equilibrium; antithixotropic systems are sols.

Eliassaf, *et al.* (2), have suggested that antithixotropy in polymethacrylic acid solutions probably results from the fact that, during flow, the collision frequency of polymer molecules increases, causing a build-up of intermolecular bonds with time. Harvey, *et al.* (3), attributed this same rheological phenomenon in pigment suspensions to a shift in

TABLE I.—DATA OBTAINED WITH THE STORMER VISCOMETER SHOWING THE INCREASE IN THE RESISTANCE TO SHEAR WITH DURATION OF SHEAR AT A CONSTANT SHEARING FORCE OF 25.4 GM.

Trial	1	2	3	4	5	6	7	8	9	10
Time of 100 rev. (sec.)	130	147	161	172	183	194	204	204	204	204
Rate of shear (sec.$^{-1}$)	46.1	40.5	37.3	34.9	32.8	30.9	29.4	29.4	29.4	29.4

TABLE II.—DATA FROM STORMER VISCOMETER DEMONSTRATING ANTITHIXOTROPY

Driving weight, Gm.	26.4	28.4	30.4	32.4	34.4	36.4	38.4	40.4
Time of 100 rev., sec.								
Upcurve	158	106	76.8	54.4	43.6	35.6	28.4	24.0
Downcurve	...	...	750	214	119	59.6	37.6	...
Upcurve	38.0	56.6	78.1	110	138	169	211	250
Downcurve	...	...	8	28	50.4	101	160	...

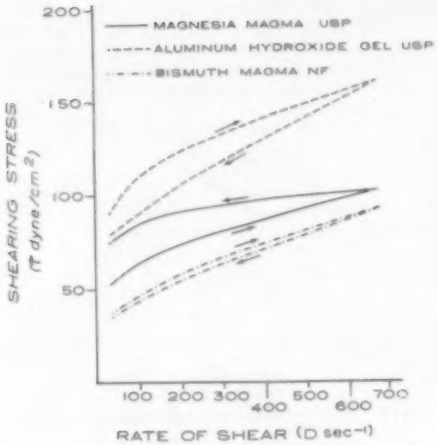

Fig. 9.—Drage rheograms for magnesia magma, aluminum hydroxide gel, and bismuth magma.

the equilibrium between two states of flocculates one having large numbers of small flocculates, and the other, small numbers of large flocculates. Both postulates probably describe similar phenomena, the second being the one most applicable to our system. From these theoretical conjectures, it would seem that the coefficient of increased resistance to shear should be some function of the rate of shear. Figure 5 indicates that with magnesia magma this is true. Also, it indicates that this coefficient is approximately equal to the square root of the shear rate used to obtain it (or is one-half order with respect to shear rate, in the kinetic sense).

Eliassaf also suggested that shear acts both in increasing and decreasing the resistance to shear. He attributed the difference between thixotropic and antithixotropic flows to a shift in the balance between these two effects. It appears from our data that a more complex situation exists in magnesia magma. At rates of shear below thirty reciprocal seconds, the flow curve indicates pseudoplastic behavior. At shear rates greater than thirty reciprocal seconds, the particle collision frequency increases to such an extent that the rate of increased resistance to shear with time becomes noticeable; moreover, the rate of decreased apparent viscosity with increasing rates of shear is relatively insensitive to the duration of shear, as shown by the parallelism of the lines of Figs. 2 and 6. This suggests that the increased resistance to shear with time and the decreased apparent viscosity with increasing rates of shear are concerned with two separate and distinct features of the system.

We suggest that these features are the floccule size and their alignment. The apparent increased resistance to shear with time at a constant shear rate results from an increase of floccule collision and a consequent change in floccule size, the change progressing until an equilibrium state is reached. On the other hand, the decreased apparent viscosity with increasing rates of shear results from greater floccule alignment in the direction of flow. With alignment there is a lower energy requirement for the bob to move through the medium than would be necessary if the increased alignment had not occurred. Further study, however, is required to clarify this rheological behavior.

The possible applications of this type of rheological behavior to product development are receiving our consideration. Also, we have under preliminary investigation, several other systems which appear to exhibit antithixotropy.

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Synthesis and Metabolic Studies of C^{14}-Labeled Hemicholinium Number Three*

By FLOYD R. DOMER† and F. W. SCHUELER

Synthesis of C^{14}-labeled hemicholinium No. 3 (HC-3) with a specific activity of 2.788 mc./mM is described. Twenty-four hour studies of rats in metabolism cages indicated 57.5 per cent of an injected dose is excreted in the urine, 15.6 per cent is excreted in the feces, and none is excreted in the expired carbon dioxide. Tissue distribution studies did not show any site of localization in rats.

In 1954 Long and Schueler (1) synthesized a series of compounds in an attempt to find a new type of anticholinesterase structure. Of special interest in this series was the compound which exhibited the lowest anticholinesterase potency. It proved to be the most toxic compound in causing death after a period of time had elapsed. A further study by Schueler (2) indicated that the compound, now called hemicholinium No. 3 (HC-3), depressed activity in the central nervous system. The potent respiratory depressant activity of this compound was attributed to its ability to form a hemiacetal configuration. Kasé and Borison (3) have shown HC-3 to have a primary site of action in the central nervous system.

HC-3 has also been found to have peripheral activity. In 1956 MacIntosh, *et al.* (4), showed that it had an effect on the rate of acetylcholine formation in the perfused superior cervical ganglion of the cat. Longo (5) recorded phrenic respiratory action potentials in rabbits and cats and obtained evidence compatible with neuromuscular blockage. Reitzel and Long (6) made an extensive study of the peripheral effects of HC-3 and found that it had neuromuscular blocking activity when the preparation being studied was stimulated fast enough to use up the stored acetylcholine. Wilson and Long (7) also found HC-3 had activity at numerous peripheral sites. It was shown to be much more potent as an inhibitor of transmission at the neuromuscular junction and parasympathetic end organs than at a sympathetic ganglion and splanchnic nerve-adrenal gland junction. Desmedt (8) demonstrated that HC-3 caused results in skeletal muscle of cats which appeared to be identical with the characteristic picture seen in patients suffering from myasthenia gravis. Orkand and Martin (9) used intracellular electrodes in the frog foot to show that high doses of HC-3 prolonged the period of depolarization and changed the shape of the end plate potential. This indicates that HC-3 may have an effect directly on the muscle in sufficiently high doses.

The preceding discussion indicates the scope of the experiments which have been carried out to show the central and peripheral actions of HC-3. Numerous studies have indicated choline to be the most effective antagonist to the action of HC-3 (2, 6, 10–12). It has been suggested that HC-3 prevents the transport of choline to the necessary intracellular sites where it is acetylated to form acetylcholine. A possible site of HC-3 action which would account for its central nervous system activity is in the blood-brain barrier where it could prevent choline transport. To test this hypothesis the present studies involve the synthesis of radioactively-labeled HC-3, its localization in various body tissues, and routes of elimination from the body.

EXPERIMENTAL

Chemical Procedures.—*Tertiary Amine Analog of HC-3.*—To 20 Gm. (71.6 m*M*) of *p,p'*-bisphenacyl chloride was added 6.45 Gm. (85.9 m*M*) N-methylaminoethanol in a flask placed in a water bath to prevent overheating. The reaction mixture was allowed to stand overnight. A 3-volume excess of water was then added and the reaction mixture allowed to stand overnight, during which time a brown precipitate formed. After washing several times with water the precipitate was collected on a filter. This product was extracted with boiling benzene and crystallized on cooling. It was repeatedly recrystallized from absolute ethanol. The melting point was 97–99° and the yield 58.4% of the theoretical amount. This agreed with the compound previously prepared in our laboratories by Dr. S. Kruger which was recrystallized from methanol. On analysis it was found to contain one mole of methanol of crystallization.

Anal.—Calcd. for $C_{22}H_{28}N_2O_4 \cdot CH_3OH$: C, 66.34; H, 7.69; N, 6.73. Found: C, 66.27; H, 7.53; N, 6.68.

C^{14}-Hemicholinium No. 3.—To 147.5 mg. (384

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† Present address: National Institute for Medical Research, Mill Hill, London, England.
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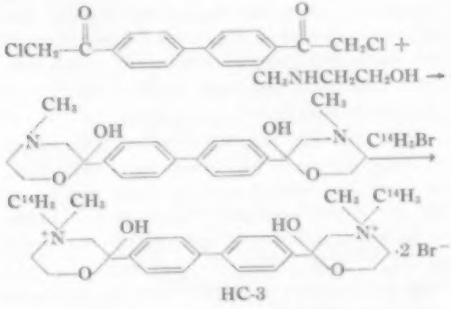
μM) of the tertiary amine thus prepared and placed in 8 ml. of 95% ethanol, 73 mg. of $C^{14}H_3Br$ (753 μM) which had been prepared in the manner described by Foreman, *et al.* (13), was added. When the addition was complete the mixture was stirred at room temperature for twelve hours. An excess of nonradioactive CH_3Br (94.4 mg.; 994 μM) was then added and the reaction stirred at room temperature for forty-one hours. Precipitation was completed by adding absolute ether and cooling in a dry ice-acetone bath. The product was filtered on a tared, medium-fritted Büchner funnel. Further recrystallization from ethanol resulted in a 95.2% yield of the theoretical of the compound which melted at 178–180°. The reaction sequence is outlined below.



HC-3

The specific activity of the compound was determined by dissolving a sample of the HC-3 in a liquid scintillator solvent containing 2,5-diphenyloxazole (PPO) and 1,4-di-[2-(5-phenyloxazolyl)] benzene (POPOP) in toluene and counting on a Los Alamos liquid scintillation counter (14). The specific activity was 2.788 mc./mM. The radiochemical purity was found by autoradiography following descending paper chromatography. Whatman No. 1 paper $1^1/_2 \times 18$ in. was used with a solvent system of *n*-butanol-ethylene glycol-water (4:1:1 by volume). The chromatogram obtained in this procedure was placed against medical X-ray film and allowed to develop for an appropriate time. The R_f of the C^{14}HC-3 in this system was 0.45.

Biological Procedures.—All animals used in these studies were male, Sprague-Dawley rats weighing approximately 250 Gm. The dose of HC-3 used was 40 mcg./Kg. i. p. which is slightly less than the LD_{50} previously reported (2).

C^{14}HC-3 was given to four rats which were immediately placed in individual all-glass metabolism cages for twenty-four hours. The use of similar cages for mice has been previously described (15). They were so arranged as to collect separately the expired carbon dioxide in a sodium hydroxide solution, urine, and feces. An aliquot of the sodium hydroxide solution was treated with an excess of a solution of 4 M ammonium chloride and 0.4 M barium chloride in CO_2-free water. The precipitated barium carbonate was collected on a medium-fritted Büchner funnel, dried in an oven, and weighed. A sample of this precipitate, along with aliquots of urine, feces, and other tissues were subjected to the procedure as has been previously described for counting of tissues in a refrigerated liquid scintillation counter (16).

A second series of animals were killed at varying periods of time after injection, i. e., twenty minutes, forty minutes, three, six, twelve, and twenty-four hours. One of each pair was then perfused via the left ventricle with about five blood-volumes of saline so as to remove the blood from the organs being studied. This is similar to the method of Schoolar, *et al.* (17). Tissues from both animals of the pair were then prepared and analyzed as in the previous section.

To study the possibility of secretion of the compound into the bile and on into the intestine, two chronic bile-fistula rats were prepared. The polyethylene cannula was run through a stab wound in the side of the animal. The animals were immobilized in Bollman-type cages and given 5% glucose in saline as drinking water and solid food, *ad. libitum*. Following a forty-eight-hour recovery period C^{14}HC-3 was injected and bile collected for two twelve-hour periods.

Autoradiographs were prepared of sections of the brain, liver, and kidneys from animals killed twenty minutes, forty minutes, and twenty-four hours after injection. Urine samples and water extracts of feces were chromatographed using the previously-mentioned *n*-butanol-ethylene glycol-water solvent system. These samples and chromatograms were then exposed to X-ray film for an appropriate length of time.

RESULTS AND DISCUSSION

The results of the twenty-four hour metabolism experiments can be seen in Table I. It will be noted that animal No. 4 shows a wide discrepancy from the other animals. Presumably, this is due to the injection having been made into the intestine instead of into the peritoneal cavity. If this animal is neglected the average results show 57.5% is excreted in the urine, 15.6% excreted in the feces, and none excreted in the expired carbon dioxide. There was no radioactivity secreted into the bile in the chronic bile-fistula animals. This, coupled with the results seen in animal No. 4 in which the HC-3 was very poorly absorbed from the intestine suggests that the HC-3 is transported into the intestinal lumen from the surrounding peritoneal cavity or bloodstream while movement in the reverse direction is much slower. This would

TABLE I.—TWENTY-FOUR HOUR EXCRETION OF C^{14}

Animal No.	Urine		Feces		CO_2	
	C^{14} d. p. m.[a]	% Inj. Dose	C^{14} d. p. m.	% Inj. Dose	C^{14} d. p. m.	% Inj. Dose
1	75,184	56.7	14,212	10.7	557	0.42
2	54,252	45.4	26,914	22.5	0	0
3	95,309	70.3	18,298	13.5	0	0
4	3,390	3.0	90,292	81.9	0	0

[a] d. p. m. = Disintegrations per minute.

TABLE II.—C^{14} TISSUE DISTRIBUTION OF TWENTY-MINUTE ANIMALS

	Unperfused			Perfused		
Tissue	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	2,628	2.4	223	0	0	0
Spleen	775	0.7	1,026	279	0.3	360
Kidneys	570	0.5	279	996	0.9	437
Muscle	0	0	0	0	0	0
Brain	420	0.4	225	183	0.2	103
Feces	3,799	3.5	2,282	2,880	2.7	1,734
Urine	546	0.5	910	0	0	0
Blood	57,312	53.1	2,274	18,631	17.2	736
Plasma	0	0	0	0	0	0

TABLE III.—C^{14} TISSUE DISTRIBUTION OF FORTY-MINUTE ANIMALS

	Unperfused			Perfused		
Tissue	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	4,955	4.2	379	3,028	2.6	221
Spleen	1,046	0.9	1,187	468	0.4	521
Kidneys	3,526	3.0	1,575	1,761	1.5	733
Muscle	...	...	218	...	...	96
Brain	408	0.3	221	60	0.1	32
Feces	3,278	2.8	1,421	15,674	13.6	3,164
Urine	40,959	35.1	45,510	14,612	12.6	22,137
Blood	38,906	33.4	1,425	23,506	20.3	871
Plasma	0	0	0	0	0	0

TABLE IV.—C^{14} TISSUE DISTRIBUTION OF THREE-HOUR ANIMALS

	Unperfused			Perfused		
Tissue	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	7,098	7.0	643	6,572	5.8	487
Spleen	959	0.9	1,391	1,833	1.6	1,660
Kidneys	2,567	2.5	1,307	1,856	1.6	714
Muscle	...	...	78	...	...	32
Brain	0	0	0	0	0	0
Feces	9,164	9.0	2,356	16,286	14.5	3,185
Urine	47,498	46.6	21,590	62,622	55.6	14,910
Blood	21,420	21.0	900	6,920	6.1	263
Plasma	0	0	0	0	0	0

TABLE V.—C^{14} TISSUE DISTRIBUTION OF SIX-HOUR ANIMALS

	Unperfused			Perfused		
Tissue	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	2,694	3.0	316	7,550	7.6	626
Spleen	325	0.4	496	835	0.8	872
Kidneys	1,936	2.1	1,108	1,584	1.6	742
Muscle	...	...	179	...	...	378
Brain	261	0.3	158	0	0	0
Feces	19,154	21.0	5,839	6,885	7.0	4,167
Urine	58,734	64.4	32,630	71,743	72.6	19,390
Blood	7,950	8.7	373	22,755	23.0	985
Plasma	0	0	0	0	0	0

TABLE VI.—C^{14} TISSUE DISTRIBUTION OF TWELVE-HOUR ANIMALS

	Unperfused			Perfused		
Tissue	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	6,757	6.1	701	2,217	2.1	189
Spleen	348	0.3	460	850	0.8	1,205
Kidneys	1,634	1.5	769	1,352	1.3	531
Muscle	0	0	0	0	0	0
Brain	0	0	0	0	0	0
Feces	30,287	27.2	5,747	26,819	25.9	10,737
Urine	78,498	70.5	12,460	63,423	61.2	23,490
Blood	25,379	22.8	976	24,424	23.6	1,009
Plasma	0	0	0	0	0	0

TABLE VII.—C^{14} TISSUE DISTRIBUTION OF TWENTY-FOUR-HOUR ANIMALS

	Unperfused			Perfused		
Tissue	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	3,015	2.7	369	1,260	1.1	110
Spleen	237	0.2	353	199	0.2	386
Kidneys	636	0.6	316	1,137	1.0	470
Muscle	...	...	203	0	0	0
Brain	0	0	0	0	0	0
Feces	90,292	79.6	17,321	26,914	22.5	3,782
Urine	3,390	3.0	295	54,252	45.4	3,487
Blood	0	0	0	18,930	15.9	678
Plasma	0	0	0	0	0	0

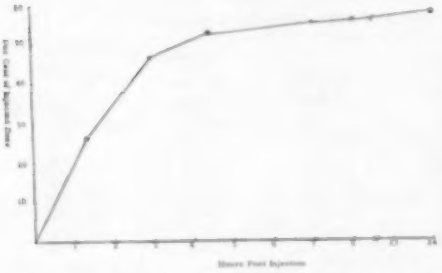


Fig. 1.—Typical urinary C^{14} excretion following C^{14} HC-3.

explain the previously-reported large increase in LD_{50} when the compound is administered orally (2). Figure 1 shows typical results obtained with urinary excretion of the radioactivity. It indicates that the compound is very rapidly excreted into the urine. Autoradiographs of urinary chromatograms showed only one compound to be present. It had a R_f of approximately 0.45 and was evidently the parent compound. This, along with the lack of respiratory $C^{14}O_2$ suggests that there is little, if any, biotransformation of HC-3 in the body.

Tables II–VII show the tissue distributions at varying times after injection. It will be noted that in most of the twenty- and forty-minute animals some of the compound had already appeared in the urine and feces. This indicates the rapidity with which the body eliminates the HC-3. Table VI shows that 87–97% of the compound was eliminated in the urine and feces in the first twelve hours following injection. The only times at which any radioactivity was found in the brain were at twenty and forty minutes after injection (the times observed to be the onset of and most severe respiratory difficulty). Autoradiographs of the brain did not show any localization of the activity. This does not indicate that the blood-brain barrier is involved in the central activity of HC-3, but it also does not preclude the possibility that it may be acting there. An interesting point which can be seen in these data is that even though there was nearly always a detectable blood level of radioactivity none was ever found in the plasma. This suggests the HC-3 was adsorbed onto or held within the formed elements of the blood. When one considers the acetylcholine-acetylcholinesterase activity of the erythrocytes this affinity is not too surprising. All of the sites of action which have thus far been found for HC-3 have been where cholinergic mechanisms exist. It will be noted that in some instances the return was greater than the injected amount of radioactivity. The most probable source of this error is the blood. In this analysis only 20λ was used as an aliquot and the resulting solution was so highly colored there was a great deal of quenching. Both of these facts would contribute to error in the determination.

Autoradiographs of the kidney showed some localization of radioactivity in the pelvis and in the cortex and/or capsule. There was no localization of radioactivity in the liver.

SUMMARY

The synthesis of C^{14}-labeled hemicholinium No. 3 (HC-3) has been described. Twenty-four hour metabolism studies with rats indicated that the HC-3 is rapidly excreted in the urine and feces (ratio approximately 3:1). Tissue distribution studies in animals at various times after injection failed to show any sites of localization in various organs of the rat.

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Notes

Note on the Chemical Components of Extracts of *Eremocarpus setigerus**

By S. NAITO and C. R. NOLLER

NUMEROUS PLANTS were used by the native California Indians as fish poisons, but the two most highly esteemed for this purpose were *Chlorogalum pomeridianum* (soap root or amole), a member of the lily family, and *Eremocarpus setigerus* Benth (*Piscaria setigera* Piper; turkey mullein, or dove weed), a member of the spurge family (1). The active principles of the former are saponins which yield sugars and steroid sapogenins on hydrolysis (2).

The common names "dove weed" and "turkey mullein" refer to the fondness of wild turkeys and mourning doves for the seeds of the plant. "Mullein" refers to the woolly, mullein-like appearance of the leaves, which is due to prickly, compound stellate hairs that radiate outward from the leaf in all directions. Some Indians attributed the killing action on fish to these hairs, which, they said, attach themselves to the eyes and gills and make the fish frantic (3). However, it was a simple matter to show that a carefully filtered aqueous extract of the leaves and the unfiltered extract were equally toxic to goldfish. It is reported also (4) that the Indians applied the bruised fresh leaves to the chest as a counter-irritant for pain and that a weak decoction of the plant was used as a bath in typhoid and other fevers and taken internally as a cure for chills and fever. In our experience, concentrated extracts have a strong vesicant action, and collecting the plants for a whole day using leather work gloves eventually led to complete loss of the outer layer of skin of the palm and fingers of the right hand without the formation of blisters.

Preliminary examination, using goldfish for toxicity tests, showed that the active principle is extractable by acetone, methanol, ether, ethylene chloride, or benzene. In the general procedure used to obtain material for chemical investigation, the ground fresh plant, collected during the month of July, was extracted with hot methanol, and the methanol was removed under reduced pressure. The residue was a mixture of the water-soluble components dissolved in the water extracted from the plant, and the water-insoluble or slightly soluble components. Extraction of the mixture with benzene gave a benzene-soluble fraction and an aqueous solution. All of the active material was in the benzene extract and none in the aqueous layer. Accordingly the active principles are not water-soluble saponins.

Removal of the benzene from the extract left a dark green sticky residue. The residue was dissolved in peroxide-free ether and chromatographed on alumina. Individual fractions were rechromatographed to give three crystalline products, which, in the order of decreasing ease of elution, proved to be paraffinic, alcoholic, and phytosterolic, none of which was toxic to fish. The melting points and analyses of these compounds agreed with those expected for hentriacontane, hexacosanol, and β-sitosterol, but the possibility that they are difficultly separable mixtures such as commonly are found in plant waxes (5) and plant sterol fractions (6) was not rigorously excluded. The more strongly adsorbed fractions were thick, sticky oils which were highly active. At a concentration of one part per million, the mean survival time of goldfish was seventy-five minutes for the crude benzene extract and twenty-two minutes after chromatographing. This toxicity may be compared with a survival time of forty-nine minutes for rotenone (7), two hundred minutes for croton oil, and thirty-five minutes for croton resin (8).

In another modification of the procedure, the residue left on evaporation of the benzene was distributed between hexane and aqueous methanol. All of the active material entered the methanol layer. Dilution of the methanol with water, extraction with ether, and evaporation of the ether left a sticky dark residue which was chromatographed on alumina. No crystalline fractions were obtained, and the toxic material appeared in the more strongly adsorbed fractions.

All attempts to obtain a single pure compound from the toxic fractions failed. Highly active fractions could be obtained by chromatography, but no crystalline product or crystalline derivative could be obtained. When a highly active fraction, separated by chromatography, was heated at 1.5 mm., a portion distilled in the range 130–185°. The portion boiling at 130–150° was inactive; that boiling at 160–185° was active, but not more so than the original fraction, and the portion distilling was small in amount.

Light-colored, highly-active fractions obtained by chromatography were free of nitrogen, sulfur, and halogen. Their infrared spectra indicated the presence of hydroxyl (2.98 μ, 3,355 cm.$^{-1}$) and carbonyl groups (5.80 μ, 1,725 cm.$^{-1}$, and 5.85 μ 1,710 cm.$^{-1}$). However, all attempts to prepare crystalline derivatives using reagents for hydroxyl and carbonyl groups failed. Refluxing with alcoholic sodium hydroxide gave a neutral fraction and an acidic fraction. Both fractions were heavy sticky oils and no crystalline derivatives could be prepared from either fraction.

Because *Croton tiglium*, from the seeds of which is obtained the highly toxic croton oil, also is a member of the spurge family, the possibility of identity or similarity of our extracts to croton oil was con-

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sidered. The literature on the chemical nature of croton oil has been summarized recently (9). In contrast to croton oil, saponification did not yield a crystalline product analogous to "phorbol" (10), nor could any crystalline esters (9) be obtained. Boehm states (11) that croton resin gives an intense red-brown color with alcoholic potassium hydroxide or sodium ethoxide, a rose red and then dark brown color on boiling with alcoholic hydrochloric acid, and a grass green color when warmed with concentrated sulfuric acid. Active fractions from *Eremocarpus* are not changed in color by alcoholic sodium ethoxide, merely darken when boiled with alcoholic hydrochloric acid, and turn a dark reddish-brown when warmed with concentrated sulfuric acid.

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Note on the Characteristics of an Apomorphine Response in Pigeons*

By A. M. BURKMAN

Quantitative relationships between the dose and incidence, latent period, duration of action, cumulative and minute responses of the apomorphine-induced pecking syndrome are described.

APOMORPHINE has long been recognized as a potent emetic in man and other animals. This substance shares with its parent morphine the property of stimulating the chemoreceptor trigger zone (CTZ) of the medulla thereby activating the vomiting center (1). CTZ excitation represents one of perhaps several nervous system stimulating components of apomorphine's spectrum of activity. The drug's emetic propensity is most readily exhibited in man and the dog, two animals who have proved to be exquisitely sensitive to this drug. Without doubt, emesis is the most apparent sign of apomorphine-induced central stimulation (following the administration of comparatively low doses) in these animals.

One sees evidence of apomorphine stimulation in a number of other animal species particularly those that are refractory to the emetic effect. In the frog, apomorphine is responsible for a marked increase in reflex excitability (2), while restlessness, excitement, and a peculiar gnawing are characteristic effects in rabbits (3). In a somewhat similar manner, the pig, rat, horse, and guinea pig also display distinct signs of stimulation (4).

The excitatory action of apomorphine manifests itself in pigeons in a manner strikingly different from that described for other animals. Following administration by any one of several routes (i. v., i. p., i. m., p. o.) this bird exhibits what has been described to be a "pecking syndrome" (5) or "feeding hallucination" (6); a continuous and repetitious pecking at the floor, walls, and roof of the cage in which it has been placed.

The relationships between the dose and a number of characteristics of this curious pecking response are herein reported.

Adult domestic pigeons of both sexes served as test subjects. All apomorphine hydrochloride solutions were administered by the intraperitoneal route, and the bird's responses monitored continuously for four hours following injections. The following observations can be reported on the basis of data contained in Figs. 1 and 2:

1. The ED_{50} (and 95% confidence limits) of apomorphine HCl, determined by the method of Litchfield and Wilcoxon (7), was found to be 0.154 (0.134–0.177) mg./Kg. while the slope function of the dose-response line (reproduced in Fig. 1) was calculated to be 1.54 (1.21–1.95).

2. Latent period following injection was observed to decrease as the dose increased. The regression line formula was calculated to be $Y = 7.39 - 2.65\,X$ (Fig. 2 *A*).

3. Pecking activity (pecks/minute) is stimulated to the greatest degree when doses of about 1.0 mg./Kg. (log dose 0) are reached (Fig. 2 *B*). Doses beyond this maximum effective dose level failed to increase the minute activity, this no doubt due to physiological limitations of the act.

4. The duration of action is an almost linear function of the log dose throughout the investigated range (Fig. 2 *C*).

5. The cumulative (total) response, as one might

* Received November 13, 1959, from the College of Pharmacy, University of Illinois, Chicago.
This project was supported by funds made available by the Research Board of the University of Illinois at the Chicago Professional Colleges.

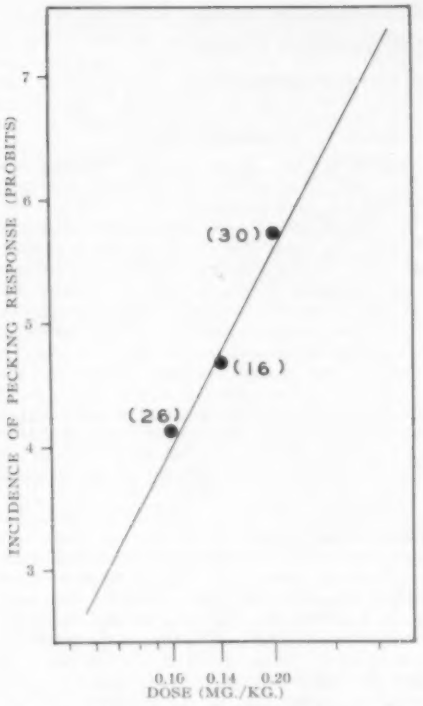

Fig. 1.—The relationship between the number of pigeons exhibiting a positive pecking response and the dose of apomorphine HCl administered intraperitoneally. Numerals in parentheses represent the total number of birds used to plot the respective points.

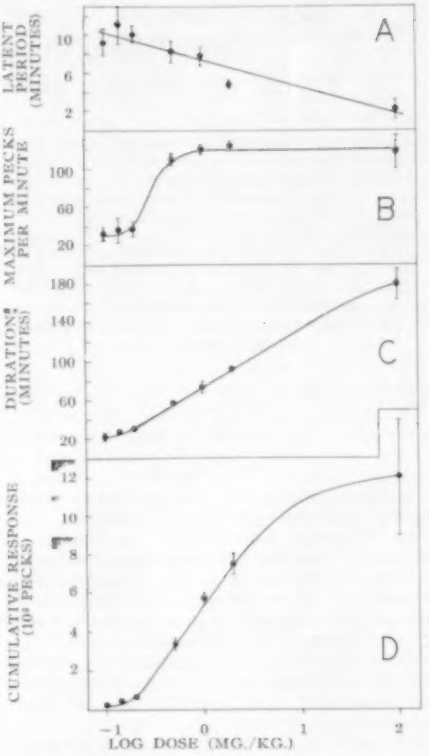

Fig. 2.—Relation of pecking syndrome characteristics to dose. Each point represents the mean (± standard error) of 12–25 pigeons.

expect after examining the above data, also increases with increase in dose. Beyond the maximum effective dose the increase in total pecking would be a direct result of increasing duration. The leveling off of the cumulative response-log dose curve (Fig. 2 *D*) at high doses warrants explanation inasmuch as the duration curve maintains a nearly continuous slope. It is suggested that this leveling off may only be an apparent statistical effect particularly since the plotted point at log dose 2 (in Fig. 2 *D*) has a rather large standard error.

Doses up to and including 100 mg./Kg. gave rise to no deleterious effects in the pigeon. Five out of a total of 66 animals exhibited from one to four brief vomiting episodes each lasting fifteen seconds or less. No response other than repititious pecking was observed in the other pigeons.

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Note on Suitable Solvent Systems Usable in the Liquid Scintillation Counting of Animal Tissue*

By GERALD A. BRUNO and JOHN E. CHRISTIAN

Animal tissues were dissolved, using hyamine base as the solvent, by several different techniques. Tissues exposed to ultrasonic excitation or homogenization upon application of heat dissolved sooner than untreated tissue. Addition of a known amount of carbon-14 standard to the tissue samples and counting of the samples by liquid scintillation techniques indicated that the methods employed for tissue solution yield satisfactory solutions for routine liquid scintillation analysis of carbon-14 in animal tissue.

WITH THE ADVENT of liquid scintillation counting in 1950, a technique especially applicable to the counting of weak beta-emitting isotopes was realized. Using the weak beta-emitting isotopes, carbon-14 and tritium (hydrogen-3), an unlimited number of compounds can be labeled and assayed with a high degree of accuracy.

This technique is particularly important to the investigator who wishes to determine the distribution and metabolism of various compounds in the animal body. With this application arises the difficulty of preparing suitable scintillator solutions of the various constituents of the animal body. The primary purpose of this investigation was to study suitable scintillator solvents for animal tissues. The instrument used was the Tri-Carb liquid scintillation spectrometer.[1]

The problem of simple preparation of animal tissue containing carbon-14 or tritium for scintillation counting, has been approached in several ways with varying degrees of effectiveness. Indirect methods reported, consist of grinding and suspending the insoluble tissue in a scintillation solvent such as toluene (1–4). The search for a simpler, more direct method of preparing clear scintillator solutions of animal tissue led to the discovery that a methanolic solution of the hydroxide form of a quaternary amine could be used to dissolve animal tissue (5). The quaternary amine chloride, commonly known as Hyamine 10X[2] (methylbenzethonium chloride) is commercially available but must undergo a relatively lengthy purification and conversion process before it can be used as a satisfactory solvent. The purified form of the Hyamine chloride is converted to the free hyamine base (methylbenzethonium hydroxide) by treatment with silver oxide (6). It has been reported that approximately 100 mg. of protein can be dissolved in 1–3 ml. of the Hyamine base (5). Since this report, several articles have appeared suggesting methods for improving the preparation procedure (7) and for modifying the method of dissolving the tissue in Hyamine (8, 9). However, no information has been reported as to the relative efficiency of detection for C^{14} in the tissue systems reported.

* Received April 4, 1960, from the Bionucleonics Dept., Purdue University, Lafayette, Ind.

[1] Model 314X, Packard Instrument Co., Inc., LaGrange, Ill.

[2] Rohm and Haas Co., Philadelphia, Pa.

EXPERIMENTAL

The optimum window for the Tri-Carb was determined to be 6–90 volts and was used in all subsequent experimentation.

The use of silver oxide as described by Passman, *et al.* (6), for conversion of the purified chloride to the base, yielded a colored solution of the base. Experimentation in this laboratory revealed that methanolic sodium hydroxide could be used to convert the chloride to the base without the formation of the highly undesirable color. A 47.8-Gm. portion of the purified Hyamine chloride was dissolved in 50 ml. of methanol and treated with 50 ml. of an 8% methanolic sodium hydroxide solution. The reaction mixture was cooled to precipitate as much sodium chloride as possible and filtered. A clear, colorless, approximately 1.0 *M* solution of the base in methanol was obtained.

Preliminary investigation using the 1.0 methanolic Hyamine base solution to dissolve rat tissue with direct heat yielded colored solutions and generally poor counting conditions. This undesirable color resulted from prolonged heating found to be necessary to solubilize the tissue samples, and the extent of the color was found to be related to the particular type of tissue being dissolved. In an effort to avoid prolonged heating, the only factor in color production which can be controlled at present, ultrasonic waves as a tissue solubilizer and homogenization procedures were employed. The accurately measured tissue samples, weighing approximately 75 mg., were placed directly in the counting vials[3] with 2 ml. of Hyamine base solution added to each. The homogenization was accomplished by breaking up the tissue with a glass stirring rod, since homogenization by other means with such small quantities was impractical. The ultrasonic wave treatment was accomplished by placing the vial containing the tissue suspended in 2 ml. of the Hyamine base solution in the transducer[4] of an ultrasonic generator and insonating it for one hour at 400 kc. Each of the samples, including an untreated sample, were heated in an oven at 100° until solution of the tissue was complete. The time for solution was noted in each instance.

The vials containing the dissolved tissue were cooled and diluted with 13 ml. of scintillator solution consisting of 4 Gm./L. of PPO and 0.1 Gm./L. of POPOP in toluene. A 20% solution of concentrated sulfuric acid in absolute ethanol was used to acidify each solution as indicated by litmus paper. This procedure eliminated the possibility of protein phosphorescence in alkaline media and thus assured the elimination of spurious counts. A known and identical amount of C^{14} standard[5] was added to each vial and the activity determined at the tap

[3] Special low-potassium 20-ml. vials with tin foil-lined screw cap, Wheaton Glass Co., Millville, N. J.

[4] Hypersonic Transducer, model Bu 301, frequency 400 kc., Brush Development Co., Cleveland, Ohio.

[5] National Bureau of Standards, beta-ray standard, carbon-14, benzoic acid in toluene, 16,500 dpm. per Gm. of solution.

setting yielding the highest count rate for each solution. The results listed in Table I indicate that the use of ultrasonics as a tissue solubilizer was advantageous, as was homogenization. In general, the heating time of the insonated tissue was less than that required by the other two methods. The results also indicate that all the tissues tested can be assayed for C^{14} with satisfactory accuracy; however, the absolute efficiency varies with the tissue involved, emphasizing the need for internal standard methods of comparison. The internal standard method makes use of the fact that upon addition of a known amount of activity one can then calculate the degree of quenching of the solution in question, thereby giving a measure of absolute activity.

TABLE I.—COMPARISON OF TIMES REQUIRED FOR SOLUTION AND CARBON-14 ABSOLUTE EFFICIENCIES OF SOLUTIONS OF VARIOUS TISSUES DISSOLVED IN HYAMINE AT 100° AND COUNTED IN THE TRI-CARB LIQUID SCINTILLATION SPECTROMETER

Tissue		Time,[a] min.	High Voltage,[b] Tap	Absolute Efficiency,[c] %
Brain	(a)[d]	25	8	36
	(b)	12	6	56
	(c)	15	6	68
Liver	(a)	60	9	24
	(b)	30	7	41
	(c)	5	7	45
Kidney	(a)	120	7	36
	(b)	15	6	59
	(c)	15	6	58
Muscle	(a)	45	6	59
	(b)	25	6	63
	(c)	20	6	61
Heart	(a)	30	9	23
	(b)	20	7	43
	(c)	20	7	54
Spleen	(a)	45	9	18
	(b)	30	7	37
	(c)	30	8	28
Intestine	(a)	60	9	42
	(b)	45	7	55
	(c)	35	7	53
Standard[e]	..	..	5	73

[a] Time in minutes required to completely dissolve tissue at the arbitrarily chosen 100° heating temperature.

[b] Tap setting which gives maximum count rate.

[c] Absolute efficiency is determined by dividing the count rate of the tissue solution by the absolute disintegration rate of the known amount of standard added to each sample.

[d] (a) Direct heating of intact tissue, (b) direct heating of homogenized tissue, (c) direct heating of tissue exposed to ultrasonics.

[e] Solution consists of N.B.S. carbon-14 standard in scintillator solution of 4 Gm./L. of PPO and 0.1 Gm./L. of POPOP in toluene.

DISCUSSION

It is important to note that results obtained for C^{14} are not wholly applicable to tritium determinations. Tissue solutions which counted with an absolute efficiency of over 50% could likely be assayed for tritium with relatively high efficiency, but the less efficient solutions which were counted at high voltage tap settings of 8 and 9, would probably be too highly quenched to be of any value in tritium counting.

To obtain an efficient scintillator solution for animal tissue, the important factors to be considered are (*a*) use of a solvent directly miscible with the organic scintillator, to minimize adulteration of the solution with solubilizing additives, and (*b*) use of whatever auxiliary means available to obtain a clear, colorless, unquenched solution.

The color produced by heating of the Hyamine base could possibly be reduced by determining the optimum temperature for tissue solution with minimum color production.

SUMMARY

1. Hyamine base was prepared by conversion of purified Hyamine chloride to the free base by treatment with methanolic sodium hydroxide. This procedure produced Hyamine base without the objectionable color previously encountered.

2. Various tissues were dissolved in Hyamine using (*a*) direct heat only, (*b*) homogenization and heat, and (*c*) ultrasonic excitation and heat. In general the tissue exposed to ultrasonic waves dissolved sooner than the untreated tissue, as did the homogenized tissue, but to a lesser extent. Reduction of heating time brought about a marked decrease in the color intensity of the samples.

3. The samples with little or no color produced during heating gave considerably higher absolute counting efficiencies than untreated tissue samples which produced highly colored solutions. Absolute efficiencies ran as high as 68% and in each instance were of sufficient magnitude to permit routine analyses.

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Note on the Constituents of the Indian Medicinal Plant *Oldenlandia corymbosa* Linn.*

By H. N. KHASTGIR, S. K. SENGUPTA, and P. SENGUPTA

γ-Sitosterol and the triterpene acids, oleanolic acid and ursolic acid, have been shown to be present in the Indian medicinal plant *Oldenlandia corymbosa* Linn. Evidence is presented to show that the plant does not contain any alkaloid.

OLDENLANDIA CORYMBOSA Linn. (Sanskrit: Parpata) of N. O. Rubiaceae is a common plant growing throughout India. The plant is used in remittent fever with gastric irritation and in nervous depression (1). A study of the plant appeared desirable because of its medicinal importance and because no chemical investigation of it had been reported in the literature. The only members of *Oldenlandia* family that have been investigated chemically are *O. auricularia*, *O. biflora*, and *O. herbaceae*. New alkaloids were reported to have been isolated from *O. auricularia* (2) and *O. biflora* (3). Later Govindachari and co-workers (4) showed that a genuine sample of *O. biflora* did not contain any alkaloid. This group of workers isolated ursolic acid from *O. biflora* and *O. herbaceae*. More recently Bhakuni (5) isolated β-sitosterol and γ-sitosterol from the seeds of *O. biflora*.

In the present study the dried plant of *O. corymbosa* was defatted with petroleum ether. From the unsaponifiable fraction of the fat γ-sitosterol was isolated and identified by the preparation of its acetate and benzoate derivatives. The defatted plant was then extracted with benzene. The residue from the benzene extract on crystallization from acetone afforded crude ursolic acid, which was purified and identified as its methyl ester and methyl ester acetate. The acetone mother liquor yielded a solid residue, which on acetylation followed by crystallization afforded oleanolic acid acetate, which in turn was further identified by the preparation of its methyl ester acetate.

The plant was finally extracted with 95% ethanol, but no crystalline material could be isolated from this fraction. Each of the petroleum ether, benzene, and ethanolic extracts gave negative tests for alkaloids.

EXPERIMENTAL

Extraction with Petroleum Ether and Isolation of the Unsaponifiable Material

Dried and crushed whole plant (2.2 Kg.) of *O. corymbosa* was extracted in a Soxhlet apparatus with petroleum ether, b. p. 60–80° for two hours. The petroleum ether was distilled off and the residual fat (20 Gm.), which showed negative test for alkaloids, was saponified by refluxing for two hours with a solution of potassium hydroxide (10 Gm.) in methanol (30 cc.) and water (2 cc.). The reaction mixture was diluted with water and the unsaponifiable material was extracted with ether, washed with water, and dried over anhydrous sodium sulfate. The removal of ether yielded the unsaponifiable material (3.5 Gm.), which was chromatographed over activated alumina (100 Gm.). Elution with ether gave a partially crystalline material (0.82 Gm.).

γ-Sitosterol.—The partially crystalline material (0.82 Gm.) obtained above was rechromatographed over activated alumina (50 Gm.), again eluted with ether, and the resulting crystalline solid (0.38 Gm.), was recrystallized from methanol to yield γ-sitosterol, m. p. 146–148°, $[\alpha]_D$ − 42.4° ($CHCl_3$). [Reported (6), m. p. 147–148°, $[\alpha]_D$ − 43°.] The sample showed a positive Liebermann test.

γ-Sitosterol Acetate.—The acetate was prepared in the usual manner with acetic anhydride and pyridine. On crystallization from methanol and then from acetone, it gave γ-sitosterol acetate, m. p. 142–143°, $[\alpha]_D$ − 46° ($CHCl_3$). [Reported (6), m. p. 143–144°, $[\alpha]_D$ − 45.3°.]

Anal.—Calcd. for $C_{31}H_{52}O_2$: C, 81.52; H, 11.48. Found: C, 81.25; H, 11.50.

γ-Sitosterol Benzoate.—The benzoate was prepared in the usual manner with benzoyl chloride and pyridine. On crystallization from acetone, it gave γ-sitosterol benzoate, m. p. 150–151°, $[\alpha]_D$ − 20° ($CHCl_3$). [Reported (6), m. p. 152°, $[\alpha]_D$ − 19.6°.]

Anal.—Calcd. for $C_{36}H_{54}O_2$: C, 83.34; H, 10.49. Found: C, 82.91; H, 10.45.

Extraction with Benzene

The defatted plant was next extracted in a Soxhlet apparatus with benzene for four hours. The removal of benzene gave a crystalline residue (9.5 Gm.), which showed a negative test for alkaloids. The residue on crystallization from acetone gave colorless crystalline solids (5.5 Gm.), m. p. 250–256°. The acetone mother liquor on evaporation gave a partially crystalline residue (*A*) (3.4 Gm.).

Methyl Ursolate.—The crystalline solids (5.5 Gm.), m. p. 250–256°, were esterified with an ethereal solution of diazomethane. The methyl ester was chromatographed over alumina (200 Gm., deactivated with 10 cc. of aqueous 10% acetic acid). Elution with a mixture of petroleum ether and benzene (3:7) afforded a crystalline solid (2.2 Gm.), m. p. 158–165°, which on recrystallization from methanol yielded methyl ursolate, m. p. 168–170°, $[\alpha]_D$ + 69.3° ($CHCl_3$). The mixed melting point with an authentic specimen[1] of methyl ursolate did not show any depression.

Methyl Ursolate Acetate.—Methyl ursolate was acetylated with acetic anhydride and pyridine in the usual manner. The acetate on crystallization from acetone afforded methyl ursolate acetate, m. p. 242–244°, $[\alpha]_D$ + 56° ($CHCl_3$). The mixed melting point with an authentic specimen of methyl ursolate acetate did not show any depression.

Anal.—Calcd. for $C_{33}H_{52}O_4$: C, 77.29; H, 10.22. Found: C, 76.83; H, 10.11.

* Received April 4, 1960, from the Research Division, East India Pharmaceutical Works Ltd., Calcutta-34, India.

The authors wish to express thanks to Mr. Priyalal Moulik for the optical rotation study and to Mr. N. Guha for encouragement.

[1] Kindly supplied by Dr. (Mrs.) A. Chatterjee, Reader in Chemistry, Calcutta University, Calcutta.

Oleanolic Acid Acetate.—The residue *A* (3.4 Gm.) from the acetone mother liquor described above was acetylated with acetic anhydride and pyridine. The crude acetate on repeated crystallization from acetone afforded oleanolic acid acetate (0.5 Gm.), m. p. 264–266°, $[\alpha]_D$ + 75° ($CHCl_3$). The mixed melting point with an authentic specimen of oleanolic acid acetate did not show any depression.

Anal.—Calcd. for $C_{32}H_{50}O_4$: C, 77.06; H, 10.11. Found: C, 76.71; H, 10.08.

Methyl Oleanolate Acetate.—The acetate was esterified with an ethereal solution of diazomethane and the ester on crystallization from acetone yielded methyl oleanolate acetate, m. p. 216–218°, $[\alpha]_D$ + 68.6° ($CHCl_3$). The mixed melting point with an authentic specimen did not show any depression.

Extraction with 95% Ethanol

The plant material after the extraction with benzene, was extracted in a Soxhlet apparatus with 95% ethanol for ten hours. The residue after the removal of ethanol showed a negative test for alkaloids and did not yield any crystalline material.

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Book Notices

Synthesis and Organisation in the Bacterial Cell. By E. F. GALE. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1959. vii + 110 pp. 12.5 × 18.5 cm.

This is a compilation of material which formed the basis of three Ciba lectures in microbial biochemistry on Structure and organization in the bacterial cell, Amino acid incorporation, and Nucleic acid and protein synthesis.

The Lynn Index. A Bibliography of Phytochemistry. Monograph IV. Organized and edited by JOHN W. SCHERMERHORN and MAYNARD W. QUIMBY. Massachusetts College of Pharmacy, Longwood Ave., Boston 15, Mass., 1960. 70 pp. 15 × 23 cm.

This monograph covers the *Glumiforae* order, including the Cyperaceae and Gramineae families. The plan for the series was described in the review of monograph I in THIS JOURNAL, **47**, 232(1958).

Précis D'Anatomie et de Physiologie Humaines. By Y. RAOUL. Masson et Cie., Éditeurs, 120 boulevard Saint-Germain, Paris 6°, France, 1960. viii + 379 pp. 16 × 21 cm. Price NF. 48,000.

This book (in French) presents a concise course in physiology for pharmacy students. It is one of a series of textbooks for the pharmaceutical curriculum.

Heterocyclic Chemistry. By ALAN R. KATRITZKY and JEANNE M. LAGOWSKI. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. 274 pp. 13.5 × 20 cm. Price $4.75.

Designed as a low-cost book for students, the authors have attempted to present a condensed version of basic heterocyclic chemistry required by graduate students and researchers. The authors have been frugal with words but liberal with almost 2,500 formulas.

Précis de Botanique. Vol. 2. Systématique des angiospermes. By P. CRÉTÉ. Masson et Cie., Éditeurs, 120 boulevard Saint-Germain, Paris 6°, France, 1960. viii + 429 pp. 16 × 21 cm. Price NF. 34,000.

This second volume on pharmaceutical botanicals (in French) considers the angiosperms (true flowering plants).

Clinical Chemical Pathology. By C. H. GRAY. Edward Arnold, Ltd., London, England, 1959. Distributed in the U. S. by the Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md. vii + 160 pp. 13 × 18.5 cm. Price $3.75.

This is the second edition of a pocket-size, handy reference on renal function, acid-base balance, fluid balance (edema) (salt and water deficiencies), liver function tests, chemical pathology of diabetes, Ca and P, disturbances of fat metabolism, chemical pathology of alimentary tract, and biochemical tests in endocrine disease. Other subjects discussed very briefly include special tests and routine clinical tests.

Antibiotics Annual 1959–1960. Proceedings of the 7th Annual Symposium on Antibiotics. Antibiotica, Inc., 30 East 60th St., New York 22, N. Y., 1960. xvii + 1034 pp. 17 × 25.5 cm. Price $15.

This volume comprises the proceedings of the seventh annual symposium on antibiotics held November 4–6, 1959, in Washington, D. C. It includes all the reports that were presented. It is, without doubt, the most comprehensive authoritative compilation of research results on new and established antibiotics for the period between the symposiums of 1958 and 1959. It is a must for reference libraries in the health sciences.

The Clinical Application of Antibiotics. Vol. 4. Erythromycin and Other Antibiotics. By M. E. FLOREY. Oxford University Press, 417 Fifth Ave., New York 16, N. Y., 1960. viii + 303 pp. 15.5 × 24.5 cm. Price $19.50.

This volume includes chapters on: Erythromycin; Antibiotics related to erythromycin by reason of bacterial cross resistance; Antibiotics which control the staphylococcus and other Gram-positive bacteria; Antibiotics inhibitory to various bacteria, including Gram-negative organisms; Antibiotics of limited clinical application owing to some toxic effect; Antibiotics active against protozoa, fungi, or neoplastic cells; and a final lengthy chapter on The choice of an antibiotic. Discussions of individual antibiotics follow the same scheme as in previous volumes: general considerations, including properties of clinical significance; antibacterial effects; evidence of toxicity; administration; and results of clinical trials. The book is an authoritative and scholarly reference with appended bibliography and index.

British National Formulary 1960. Standard Edition. The British Medical Association and the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, 17, Bloomsbury Square, London, W. C. 1., England. Obtainable from Rittenhouse Book Store, 1706 Rittenhouse Square, Philadelphia 3, Pa., 1960. 272 pp. 10.5 × 16.5 cm. Price 7s. 6d. Interleaved, 11s. 6d.

This is the fifth edition of the pocket-size prescribers' formulary. Additions are listed by B. N. F. title and the proprietary name of an equivalent or similar preparation is given.

Gehes Codex. 9th ed. Edited by FELIX DIEPENBROCK. Wissenschaftliche Verlagsgesellschaft M. B. H., Stuttgart, and Schwarzeck-Verlag G. M. B. H., Munchen, Germany. Distributed in the U. S. by Texstar Co., 200 West 34th St., New York 1, N. Y., 1960. xi + 1392 pp. 15 × 23 cm. Price $38.25.

This ninth edition of "Gehes Codex" continues the exceptional coverage of new drug products on the world market, with particular attention to European products, that users of earlier editions have come to expect. An added feature in the latest edition is the indication of trade-marked names. No reference library in pharmacy can be considered complete without the presence of the latest "Gehes Codex."

Virus Virulence and Pathogenicity. By Ciba Foundation Study Group No. 4. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1960. viii + 114 pp. 12 × 18.5 cm.

This small book includes reports on: The definition and measurement of virus virulence, Host-cell factors and virus virulence, The effect on virulence of changes in parasite and host, Broad aspects of human virulence in influenza viruses, The severity of influenza as a reciprocal of host susceptibility, and The virulence for man of some respiratory viruses passed in tissue cultures. A general discussion and an index are included.

Basic Facts of Body Water and Ions. By STEWART M. BROOKS. Springer Publishing Co., Inc., 44 East 23rd St., New York 10, N. Y., 1960. 159 pp. 14 × 21 cm. Paperbound. Price $2.75.

The body's fluid and electrolyte balance in health and in disease states is concisely presented under the two main parts: The facts explained and The facts applied. A glossary and index are appended.

Handbook of Filtration. 1st ed. By the technical staff of the Eaton-Dikeman Co. The Eaton-Dikeman Co., Mt. Holly Springs, Pa., 1960. viii + 124 pp. 15.5 × 23.5 cm. Price $2.50.

This book by the technical staff of The Eaton-Dikeman Co. covers historical development of filtration, paper test methods, retention, permeability, and applications of filter paper. A glossary and index are appended.

Textbook of Pharmaceutical Chemistry. 7th ed. By J. E. DRIVER. Oxford University Press, 417 Fifth Ave., New York 16, N. Y., 1960. ix + 728 pp. 15.5 × 24.5 cm. Price $14.50.

While still maintaining its original design as a student's textbook, the new seventh edition of Bentley and Driver's (Driver sole author since 2nd ed.) is a useful reference for practicing pharmacists and chemists. Part I gives physical and chemical methods of assay for pharmaceutical substances, with detailed applications given in Parts II and III. Part II covers the inorganic materials and Part III is devoted to organic compounds of pharmaceutical importance.

Methoden der Organischen Chemie (Houben-Weyl). 4th vol. of the new series. Band V/4, Halogenverbindungen. Edited by EUGEN MÜLLER. George Thieme Verlag, Herdweg 63, Stuttgart-N, West Germany, 1960. Agents in U. S. and Canada, Intercontinental Book Corp., 381 Fourth Ave., New York 16, N. Y. xlvii + 894 pp. Price DM 180, $42.85.

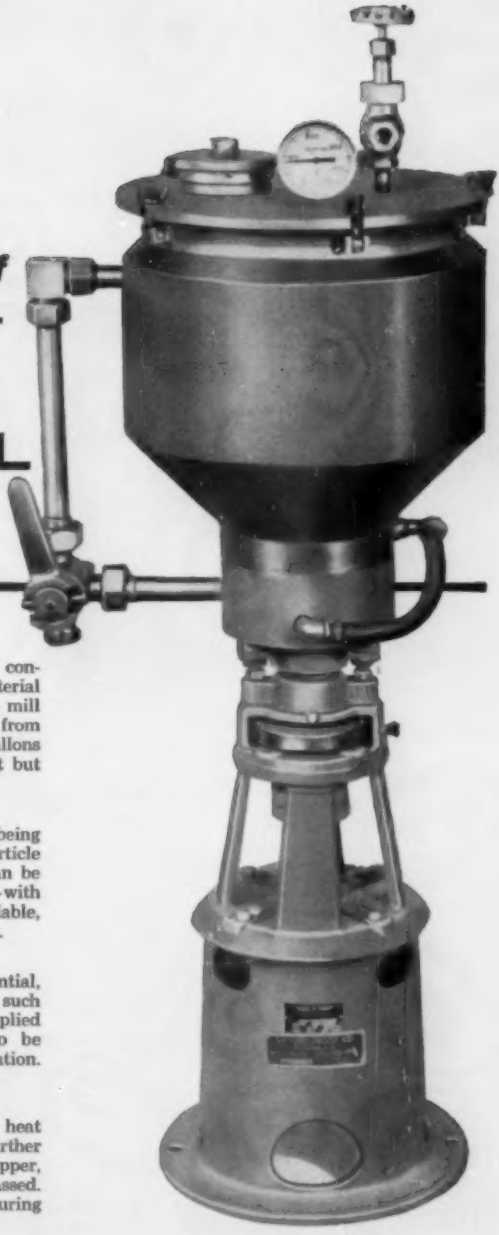
This volume of a long recognized standard reference series takes up the halogen compounds. The preparation of bromine and iodine compounds and the reactivity and transformation of chlorine, bromine, and iodine compounds are covered. This comprehensive treatise abounds with references to the literature throughout the text, and author and subject indexes are included.

Chemical Analysis. Vol. 12, Systematic Analysis of Surface-Active Agents. By MILTON J. ROSEN and HENRY A. GOLDSMITH. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. xvii + 422 pp. 15 × 23 cm. Price $13.50.

This volume treats of a growing and complex group of materials. Surface-active agents are classified and then discussed under the sections: Detection, isolation, and estimation in compositions; Qualitative analysis; Quantitative analysis; Separation of mixtures of surface-active agents. A table of representative commercially-available surfactants is appended and a subject index is included.

For Rupturing Large Quantities of Microorganisms—

The Eppenbach MICRO-MILL



The *G-W Eppenbach Micro-Mill* is specially constructed for the reduction of slurries and bacterial spores, molds, yeast and pollen granules. The mill functions perfectly with hopper capacities ranging from 150 ml to 2 liters of slurry. Capacities up to 5 gallons are available. The Micro-Mill is not a test unit but has proved successful in actual operation.

FLEXIBLE, PRECISION CONTROLS

120 micron glass beads mix with the slurry being examined. A calibrated dial provides exact particle breakdown settings. And rotor-stator settings can be precisely designated from 0.0005 to 0.125 in.—with both bead-cell ratio and mill gap settings variable, anytime, to produce optimum, critical reductions.

SPECIAL ENVIRONMENTAL CONDITIONS

For maintaining anaerobic conditions where essential, the Micro-Mill's recirculating system establishes such an environment. The stainless hopper cover is supplied with a needle valve enabling the system to be blanketed with a suitable gas to minimize oxidation.

CRITICAL TEMPERATURES ACCOMMODATED—AND CONTROLLED

Externally jacketed to accommodate passage of heat transfer fluids, media temperature controlling is further assisted by a stainless coil located inside the hopper, through which heat transfer fluids may be passed. Included are a thermowell and thermometer assuring pin-point readings throughout the stages.

1.5 HP AC-DC VARIABLE SPEED MOTOR

The Micro-Mill is powered by a 1.5 hp AC-DC motor with speed controls from 0 to 10,000 rpm monitored by a continuously variable transformer, and mounted on stainless dolly for portability. Also available with integral refrigeration unit. These and other quality features make the easy-to-use, easy-to-install—and clean—Eppenbach Micro-Mill an outstandingly valuable piece of equipment. Write for complete details.

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Hudson, N. Y. • Since 1814

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For quick and prolonged relief

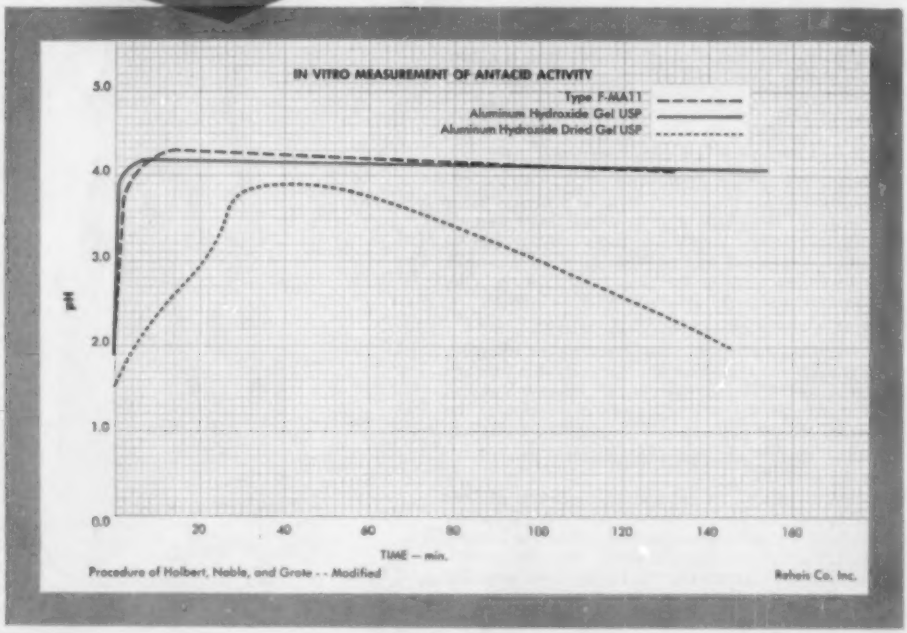
Aluminum Hydroxide-Magnesium Carbonate Co-Dried Gel Type F-MA11

A finely-divided, white powder consisting of a combination of aluminum hydroxide gel and magnesium carbonate, prepared by a new process* that yields a gastric antacid in dry form having extremely prompt and very prolonged acid-neutralizing action in the optimum pH range.

The ideal properties of this product, especially suited for the ambulatory patient, are not lessened by aging nor by pepsin present in gastric juice.

Full data and samples of this newest Reheis antacid and buffer compound are available on request.

*U.S. Patent No. 2,797,978



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